Role of IL-3 receptor alpha isoforms and microRNAs in IL-3-driven myeloid differentiation

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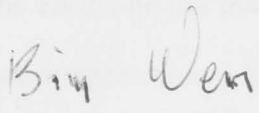
Statement of Originality

This thesis contains no material that has been accepted for an award of any degree or diploma by other universities. The materials presented in this thesis are, except where otherwise reference has been made, my own original work.



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Abstract

Blood cell formation involves proliferation of hematopoietic stem cells coupled with progressive maturation and lineage commitment. There is a critical requirement to maintain stem cell numbers by self-renewal. The mechanisms which control both differentiation and the balance between self-renewal and differentiation are poorly understood. The focus of this thesis is on myeloid differentiation. Four myeloid differentiation pathways have been recognized: (1) steady state myeloid differentiation, which maintains the normal levels of mature myeloid cells, (2) cytokine-driven or inducible myelopoiesis which generates additional myeloid effector cells during infections or allergic responses, (3) *All-trans* retinoic acid (ATRA)-induced differentiation of promyelocytic leukemia cells and (4) CD44-driven differentiation of AML blasts. The aim of the thesis work was to gain a better understanding of IL-3-driven myeloid differentiation.

In Chapter 3, investigations of the hIL-3R system in the promyelocytic leukemia line NB4 are presented. NB4 is not only blocked in steady-state granulocytic differentiation but also in cytokine-induced differentiation driven by IL-3 or GM-CSF. The IL-3R system was present at low levels in NB4 but was otherwise normal. Elevation of hIL-3R levels by ectopic expression restored IL-3-induced differentiation, but differentiation was only partial indicating that the PML-RARα protein also interferes with the induction of IL-3-driven differentiation. SiRNA knockdown of hβc did not affect ATRA-driven differentiation.

In Chapter 4, studies relating to the CD44-induced differentiation pathway are presented. Although it is known that CD44 is a potent inducer of differentiation of promyelocytic leukemia cells, siRNA knockdown of CD44 did not affect ATRA-induced differentiation of NB4 cells and also did not affect IL-3-induced differentiation.

In Chapter 5, the development of a new *in vitro* model for studying the mechanisms of mIL-3-induced myeloid differentiation is presented which utilizes the conditionally immortalized GM progenitor SCF ER-Hoxb8. The data indicate that IL-3-induced differentiation results in significant upregulation of miR-223 and the transcription factors c/EBPε and C/EBPβ. Additionally, the data show an important role for signaling via the IL-3Rα SP2 isoform in the differentiation of this immortalized GM progenitor.

Collectively, the thesis results indicate that the mechanisms involved in the induction of differentiation via the IL-3-driven, steady state, ATRA-induced and CD44-induced pathways are separate but they likely invoke a common granulocytic differentiation pathway which involves upregulation of C/EBPε, C/EBPβ and miR-223. The new IL-3 differentiation model provides the foundation for future studies which could lead to miRNA directed strategies for controlling allergic inflammation and a better understanding of the pathogenesis of AML.

Abbreviations

Ab - Antibody

Ag - Antigen

AHR - Airway hyperreactivity

ALL - Acute lymphoblastic leukemia

Amino acid - AA

AML - Acute myeloid leukemia

AMOs - Anti-miRNA oligonucleotides

AP-1- Activator protein 1

APL - Acute promyelocytic leukemia

ATF2 - Activating transcription factor 2

ATRA - *All-trans* retinoic acid

BaPs - Basophil lineage-restricted progenitors

BM- Bone marrow

BMCPs- Basophil/mast cell progenitors

Bp - Base pair

BSA- Bovine serum albumin

βc - Beta common receptor chain

CBAP - Common beta-chain associated protein

CD11b - Cluster of differentiation antigen 11b

CD15 - Cluster of differentiation antigen 15

CD44v - CD44 splice variants

CD44s - CD44 standard

cDNA - Complementary DNA

C/EBPs - CCAAT/enhancer-binding proteins

CIP - Calf intestinal alkaline phosphatase

CML - Chronic myeloid leukemia

CMPs - Common myeloid progenitors

CRM - Cytokine-receptor homology module

Ct - Real-time PCR threshold cycle

DCs - Dendritic cells

Dg - Deglycosylation

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

dsRNA - Double stranded RNA

EoPs- Eosinophil lineage-restricted progenitors

EPO - Erythropoietin

EPOR - Erythropoietin receptor

ER - Estrogen receptor

ERK- Extracellular signal-regulated kinase

FACS - Flow cytometry

FBS - Fetal Bovine Serum

FCS - Fetal Calf Serum

FcRγ - Fc receptor common γ-chain

FLT3 - FMS-like tyrosine kinase 3

GATA-1 - GATA binding protein 1 (globin transcription factor 1)

G-CSF - Granulocyte colony-stimulating factor

GFI1 - Growth-factor independent 1

GH- Growth hormone

GHR- Growth hormone receptor

Glu - Glucagon

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GMPs - Granulocyte/macrophage progenitors (GM progenitors)

HA - Hyaluronic acid

Hβc - Human beta common receptor

HPRT - Hypoxanthine-guanine phosphoribosyl transferase

HSC - Hematopoietic stem cells

IFN-β - Interferon-beta

IgE - Immunoglobulin E

IKK - IκB kinase

IL-3 - Interleukin 3

IL-4 - Interleukin 4

IL-5 - Interleukin 5

IL-12 - Interleukin 12

IL-3Rα - Interleukin-3 receptor alpha

IL-3Rs - Interleukin-3 receptor system

IRFs - Interferon-regulatory factors

ITAM - Immunoreceptor tyrosine-based activation motif

JAK2- Janus kinase2

JNK - c-Jun N-terminal kinases

LEF1 - Lymphoid enhancer-binding factor 1

LSC - Leukemic stem cell

MAPK - Mitogen-activated protein kinase

Mβc - Mouse β common receptor

$m\beta_{IL\text{-}3}$ - Mouse IL-3 specific β receptor

M-CSF - Macrophage colony-stimulating factor

MEK - MAPK or Erk kinases

miRNA - microRNA

mRNA- messenger RNA

Multi-CSF - Multi-lineage colony stimulating factor

Nb - Nippostrongylus brasiliensis

NFI-A - Nuclear factor 1 A-type

NF-κB - Nuclear factor-κB

NK cells - Natural killer cells

NOD/SCID - Nonobese diabetic/ severe-combined immunodeficient

OD - Optical density

OPN - Osteopontin PI3-K -Phosphatidylinositol 3-kinases

PAP - Pulmonary alveolar proteinosis

PBS - Phosphate buffered saline

PCR - Polymerase Chain Reaction

PIP_3 - Phosphatidylinositol-3,4,5-triphosphate

PLZF - Promyelocytic leukaemia zinc finger

PML - Promyelocytic leukemia

PNGase F - Peptide *N*-glycosidase F

PTGS - Post-transcriptional gene silencing

RA - Retinoic acid

RARα - Retinoic acid receptor alpha

RARs - Retinoic acid receptors

RISC - RNA-inducing silencing complex

RNA - Ribonucleic acid

RNase - Ribonuclease

RNAi - RNA interference

RT - Reverse transcription

RUNX1 - Runt-related transcription factor 1

SCF - Stem cell factor

SCL - Stem-cell leukemia factor

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser - Serine

SH2 - Src homology 2

SHC - Src homology and collagen

SHP - Src homology protein

siRNA - Small interfering RNA

SOS - Son of sevenless

SOCS - Suppressors of cytokine signaling

SPP1- Secreted phosphoprotein-1

STAT - Signal transducer and activator of transcription

TCF - Ternary complex factor

Th1 cells - Type 1 helper T cells

Th2 cells - Type 2 helper T cells

Thr - Threonine

TPO - Thrombopoietin

Tyr - Tyrosine

WSXWS - W = tryptophan, S = serine, and X = any amino acid

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Chapter 1

Introduction

The work described in this thesis relates to investigations of the mechanisms of IL-3-induced myeloid differentiation. These are poorly understood at present but are relevant to allergic inflammation and to acute myeloid leukemia. In order to introduce this topic this Chapter gives an overview of hematopoiesis, the biology of IL-3 and GM-CSF and their receptors, the relevant signaling pathways and the mechanisms regulating myeloid differentiation.

1.1 Hematopoiesis

Hematopoiesis is the process by which blood cells are derived from multipotent stem cells that are found in the bone marrow in the adult. During foetal development, hematopoiesis begins in the blood islands of the yolk sac and then develops in the liver, spleen and lymph nodes before the bone marrow becomes the primary site for hematopoiesis throughout life (Gallicchio, 1998). Multipotent stem cells can proliferate in response to appropriate stimuli to produce large numbers of progenitor cells that become committed to one or another of the major cell lineages of the blood. In the steady state the numbers of progenitor cells is small, but this compartment can be rapidly expanded under conditions of increased demand, such as infection or bleeding, or during growth of the individual. Under these conditions, alternative sites of hematopoiesis may be utilised, including the liver and spleen. This process is referred to as extramedullary hematopoiesis (Gallicchio, 1998).

Hematopoietic stem cells are referred to as multipotent because they have the ability to differentiate into mature cells of both the myeloid and lymphoid lineages, and are also capable of self-renewal (Spangrude *et al.*, 1988; Spangrude, 1991). Importantly, hematopoietic stem cells are capable of engrafting into a myeloablated adult and reconstituting all lineages of the hematopoietic system (Spangrude *et al.*, 1988; Moore and Sakamoto, 2005). While stem cells are multipotent, committed progenitor cells

differentiate into a limited number of different cell lineages. As progenitor cells undergo further commitment, their proliferative and lineage potential is reduced until they finally form one of the mature blood cell types.

Although the mechanisms regulating hematopoiesis are poorly understood, it is well established that this process can be stimulated by cytokines. The colony-stimulating factors such as IL-3 and GM-CSF stimulate multilineages of hematopoietic cells, while IL-5, G-CSF, M-CSF, erythropoietin (EPO) and thrombopoietin (TPO) stimulate more restricted lineages (Heimfeld *et al.*, 1991). IL-3 can positively regulate the proliferation and differentiation of hematopoietic stem cells. After binding to its receptor on target cells, IL-3 promotes proliferation and differentiation of hematopoietic stem cells and the survival of mature cells.

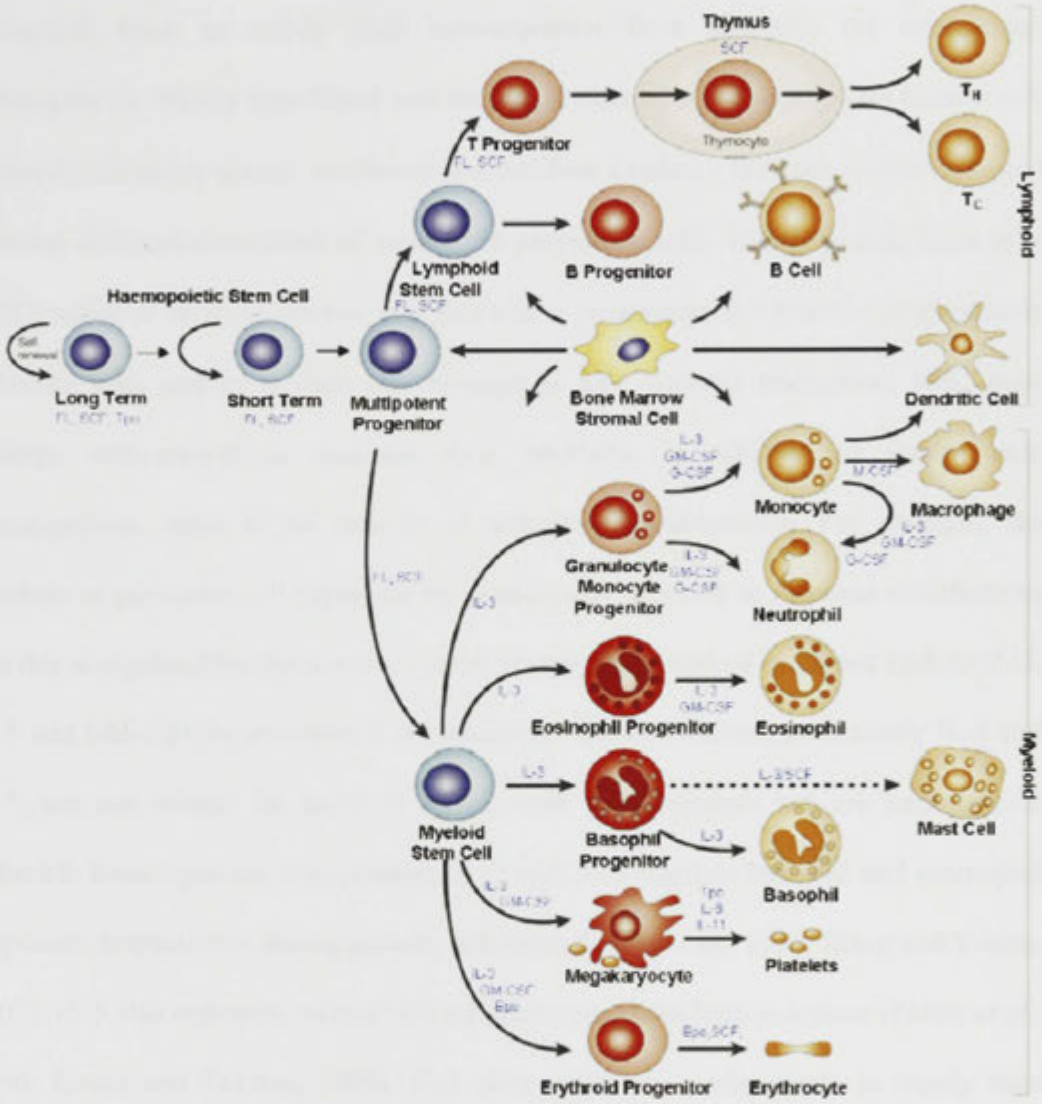


Figure 1.1: Schematic representation of hematopoiesis (adapted from Nabel, 2002). The cytokines stimulating stem cell self-renewal and lineage commitment are shown.

1.2 Basal versus inducible hematopoiesis

In considering the role of cytokines in regulating hematopoiesis, it is important to distinguish basal or steady state hematopoiesis from inducible (or emergency) hematopoiesis. Steady state blood cell formation maintains the appropriate basal levels of blood cells under normal conditions and involves a balance between proliferation and terminal differentiation/death of committed progenitor cells. Hematopoietic stem cells (HSC) reside in the bone marrow and give rise to progenitors that become progressively restricted with respect to their cell lineages as they undergo maturation. HSC must undergo self-renewal to maintain their numbers. In addition to steady state hematopoiesis, there is the process of inducible hematopoiesis. For example, the numbers of particular cell types can be dramatically elevated in response to infections and this is regulated by the immune system via the production of cytokines such as IL-3, IL-5 and GM-CSF by activated T lymphocytes. These cytokines, particularly IL-3 and IL-5, are not critical for basal or steady state hematopoiesis but are important in inducible hematopoiesis. For example, IL-3 and IL-5 regulate basophil and eosinophil responses, respectively, during parasite infections (Lantz *et al.,* 1998; Wang and Young, 2007). IL-5 also regulates eosinophilic inflammation of the lung in asthma (Foster *et al.,* 1996; Kouro and Takatsu, 2009). IL-3 does not *have a* unique role in steady state hematopoiesis but it does in relation to basophils and, to a lesser extent, mast cells in inducible hematopoiesis as discussed in detail below.

The control of differentiation versus self-renewal is not well understood but some general principles have been established. Hematopoietic stem cells (HSC) reside in the bone marrow and give rise to progenitors that become progressively restricted with respect to their cell lineages as they undergo maturation. Specific transcription factors are induced which control lineage determination during differentiation (Zhu and

Emerson, 2002) in conjunction with miRNAs which appear to provide an additional level of control (Fatica *et al.,* 2008). In addition, the HSCs must undergo self-renewal to maintain their numbers. Hox homeodomain transcription factors promote self-renewal and expansion of hematopoietic progenitors and are down-regulated during differentiation and maturation (Owens and Hawley, 2002). They are commonly dysregulated in mouse and human acute myeloid leukemia (AML). The mechanisms which control both differentiation and the balance between self-renewal and differentiation are critical to maintaining normal blood cell development. Their failure leads to diseases like leukemia in which there is excessive self-renewal and defective differentiation.

1.3 Leukemia

Leukemia involves an uncontrolled proliferation of progenitor cells in the absence of differentiation. Uncontrolled proliferation can arise through changes that lead to an increased proliferative capacity of the progenitor cell and/or a block in differentiation (Kuby, 1997). A block in differentiation results in an accumulation of immature cells that fail to fully mature and subsequently die. Proliferating immature cells that are blocked in their differentiation and escape immune surveillance, eventually dominate the bone marrow and invade other tissues and organs leading to death (Kuby, 1997; Evans *et al.,* 1998).

While the processes of proliferation and differentiation are central to the development of leukemia, apoptosis is also involved in the control of leukemia. Homeostasis is maintained by balancing cell proliferation with cell death and an imbalance in either may result in cancer (Kuby, 1997). Cytokines can promote proliferation and tumour suppressor genes can inhibit proliferation. The regulation of apoptosis can occur by mutation of genes that either block or induce apoptosis. For example, in B-cell follicular

lymphoma a chromosomal translocation of the anti-apoptotic gene bcl-2 occurs leading to its over-expression and inhibition of apoptosis (Golub *et al.,* 1999). Inhibition of apoptosis enables a leukemic cell to escape its usual programmed cell death and can enable the cell to live almost indefinitely. However, other events that enable the cell to proliferate and maintain its self-renewal capacity must also occur for immortalisation of a cell. As stated above, a block in differentiation can also lead to leukemia. In myeloid leukemias, chromosomal translocations occurring in genes involved in the regulation of transcription are a common mechanism leading to arrested differentiation (Warner *et al.,* 2004). Warner *et al.,* (2004) suggest that "alterations in transcriptional regulation leading to aberrant myeloid differentiation may be a necessary step in leukemogenesis," but are not sufficient for progression to acute myeloid leukemia (AML) without additional events providing proliferative and survival advantages.

In human leukemia, gross chromosomal changes occur quite frequently resulting in the fusion of unrelated genes creating chimeric proteins with altered functions (Brendel and Neubauer, 2000). In many cases, the disruption of each gene's normal function by the formation of the novel chimeric protein has been demonstrated to be responsible for the disease (Pandolfi, 2001). The mutations that cause leukemia fall into two broad groups. Mutations in the first group provide a proliferative or survival advantage on the hematopoietic cells by the aberrant activation of signal transduction pathways (Gilliland *et al.,* 2004). In contrast, mutations in the second group inhibit differentiation or provide self-renewal properties to the hematopoietic cells during development through the mutation of transcription factors or transcription co-activators that mediate their effects on cell differentiation or self-renewal (Gilliland *et al.,* 2004).

It was shown that concomitant abnormal expression of the transcription factor Hoxb8 and the hematopoietic growth factor IL-3 generates a rapid-onset transplantable myeloid

leukemia in mice (Ymer *et al.*, 1985; Perkins *et al.*, 1990). Hoxb8 appears to immortalize an early hematopoietic progenitor by blocking its differentiation. Continuous IL-3 dependent lines are readily created from Hoxb8-immortalized progenitors but require unusually high levels of IL-3 compared with other IL-3-dependent cell lines. This property originally led to the suggestion that another form of the IL-3 receptor may exist in the immortalized progenitors but it has remained unexplained (Perkins and Cory, 1993). The special relationship between Hoxb8 and IL-3 in the generation of AML in mice makes it critical to elucidate how Hoxb8 blocks differentiation and to uncover the special nature of IL-3 signaling in Hoxb8-immortalized progenitors.

1.4 Biology of IL-3, GM-CSF and IL-5

The work described in this thesis is primarily concerned with IL-3-supported myeloid differentiation, but it is useful to compare the biology of IL-3 with the biology of the related cytokines, GM-CSF and IL-5 which share the same receptor system. In addition, GM-CSF is used in some of the studies for comparison of its signaling effects with those of IL-3.

1.4.1 IL-3 biology

IL-3, also referred to as multi-lineage-colony stimulating factor (Multi-CSF), is produced mainly by activated $CD4^+$ T cells and mast cells. IL-3 acts on the most immature marrow progenitors (Metcalf, 1989) and is a multipotent cytokine which promotes myeloid progenitor cell self-renewal and differentiation, and the survival and activation of mature myeloid cells (Spivak *et al.*, 1985; Metcalf *et al.*, 1986; Wagemaker *et al.*, 1990). IL-3 is a critical mediator of inflammation and immune responses to infections. IL-3-deficient mice show a defective capacity to elevate mast

cell and basophil numbers in response to parasite infections (Lantz *et al.,* 1998). It is important to note that IL-3 does not play a critical role, however, in basal hematopoiesis.

IL-3 can promote the growth and differentiation of $CD34^+$ progenitor cells into basophils and mast cells, myeloid-derived dendritic cells and non-myeloid derived dendritic cells, and to a lesser extent, eosinophils and monocytes-macrophages (Lantz *et al.,* 1998; Ebner *et al.,* 2002; Strobl, 2003). This pattern of IL-3 responsiveness corresponds to the cellular expression of IL-3Rα (Yamada *et al.,* 1998). IL-3, but not IL-5 or GM-CSF, prevents basophil apoptosis *in vitro* through the activation of PI3-K (Zheng *et al.,* 2002). IL-3 also promotes stem cell factor–induced mast cell differentiation in a dose-dependent manner and enhances FcεR1-dependent mediator release (Gebhardt *et al.,* 2002). The role of IL-3 in relation to basophils and Th2-type allergic responses is discussed in more detail below.

A potential role for IL-3 in the pathophysiology of inflammation in the central nervous system has been suggested (Araujo and Lapchak, 1994; Appel *et al.,* 1995). Data to support this notion include the observation in rats that microglia can produce and respond to IL-3 and that βc expression in the brain is restricted to the microglial cells, suggesting that IL-3 might function in an autocrine feedback loop (Appel *et al.,* 1995). *In vitro* IL-3 stimulation of microglial cells induced their proliferation and the formation of multinucleated giant cells (Lee *et al.,* 1993). The clinical relevance of these observations is suggested by the detection of IL-3 in postmortem brain tissue from patients with Alzheimer's disease (Araujo and Lapchak, 1994). Taken together, these data suggest that IL-3 might mediate increased activation of microglial cells and the subsequent development of neurodegeneration.

The role for IL-3 in modulating type 1 dendritic cells (DCs) function was also reported (Ebner *et al.,* 2002). When the human type 1 dendritic cells were derived *in vitro* with IL-3 rather than GM-CSF, the dendritic cells preferentially induced Th2 responses. In contrast, type 1 dendritic cells derived with GM-CSF preferentially induced Th1 responses. This difference was the consequence of decreased IL-12 production by IL-3–derived type 1 dendritic cells. In addition, differentiation of type 2 dendritic cells requires IL-3, and these cells also produce low quantities of IL-12 and promote Th2 responses (Ebner *et al.,* 2002). Thus IL-3 might become an important molecule in the development of strategies to modulate Th1 and Th2 immune responses. IL-3 is the key cytokine for generating DCs from high IL-3R expressing plasmacytoid precursor cells, by ensuring their proliferation and survival. However, IL-3Rs are also expressed, albeit at lower levels, in monocytes (Rissoan *et al.,* 1999), and IL-3 has been reported to play a role in the generation of DCs when using blood monocytes as precursors. In particular, it has been demonstrated that IL-3 can replace GM-CSF and, combined with either IL-4 or IFN-β, leads to the generation of DCs endowed with distinct functional properties (Buelens *et al.,* 2002; Ebner *et al.,* 2002).

IL-3 released by infiltrating inflammatory cells in different pathological settings contributes to organ and tumor angiogenesis. IL-3 expands a subset of $CD45^+$ circulating angiogenic cells clonally derived from the hematopoietic progenitors. Moreover, $CD45^+$ cells exposed to IL-3 acquire arterial specification and contribute to the formation of vessels *in vivo*. In inflammatory microenvironments containing IL-3, angiogenic cells derived from hematopoietic precursors can act as adult vasculogenic cells (Zeoli *et al.,* 2008).

The expression of IL-3Rα on a wide range of hematological malignancies is of potential importance as a marker of tumor load (Munoz *et al.,* 2001). Although not normally

expressed by B cells, increased expression of IL-3Rα is observed in approximately 40% of patients with B cell-acute lymphocytic leukemia or acute myeloid leukemia. Furthermore, the increased expression of IL-3Rα is associated with enhanced blast proliferation, increased cellularity, and a poor prognosis (Testa *et al.,* 2002). The IL-3R represents one of the target molecules involved in the stimulation of proliferation of AMLs, and the overexpression of the IL-3Rα chain may represent one of the mechanisms contributing to the development of a highly malignant leukemic phenotype (Testa *et al.,* 2004). The studies on the IL-3Rα have indicated it is an important marker in leukemia and leukemic stem cells. The new anti-leukemic drugs targeting the IL-3Rα are now under development (Testa *et al.,* 2004).

1.4.2 IL-3, basophils and type 2 immunity

Basophils are rare blood granulocytes that are generated in the bone marrow from their progenitors and enter the circulation as fully mature cells (Falcone *et al.,* 2000). Basophil numbers are increased during parasite infections and in allergic inflammation (Luccioli *et al.,* 2002). The role of IL-3 in allergic diseases is supported by its *in vitro* effects on eosinophils and basophils. Basophils are postulated to be the circulating effector cells of allergy. IL-3 has been shown to directly cause the release of histamine from basophils of allergic asthmatic subjects (Sugiyama *et al.,* 1993). Furthermore, recruitment of basophils to sites of allergic inflammation may also be increased by IL-3, mediated via the up-regulation of the adhesion molecule CD11b, the α-chain of the complement receptor type 3 (mac-1) (Bochner *et al.,* 1990).

The mechanisms involved in induced basophil generation are poorly understood but there is much evidence of an important role for IL-3. For example, incubating bone marrow cells with IL-3 *in vitro* promotes their differentiation into basophils (Yoshimoto *et al.,* 1999; Gessner *et al.,* 2005). *In vivo*, basophilia can be induced by IL-3 treatment

of mice and parasite-induced basophilia is due to increased endogenous production of IL-3 (Oh *et al.,* 2007). Treating Nippostrongylus brasiliensis (Nb)-infected mice with neutralizing anti-IL-3 antibody partially diminished basophil accumulation in the periphery (Min *et al.,* 2004). Voehringer *et al.,* (2004) reported that administration of the IL-3 complex (IL-3 plus anti-IL-3 Ab) *in vivo* promoted the differentiation of GMPs into basophil lineage-restricted progenitors (BaPs) in a STAT5 signaling-dependent manner and increased the number of basophil/mast cell progenitors (BMCPs) in the spleen. IL-3 receptor expression was dramatically up-regulated in BaPs, but not in eosinophil lineage-restricted progenitors (EoPs). It has been shown that about 38% of BMCPs express the IL-3 receptor (Ohmori *et al.,* 2009). IL-3 induces basophil expansion by promoting granulocyte-monocyte progenitors and basophil-mast cell progenitors to differentiate into basophil lineage progenitors (Ohmori *et al.,* 2009; Arinobu *et al.,* 2005). It has been reported that activated T cells are the major source of IL-3, which acts to enhance basophil generation in the bone marrow (BM) during parasite infection. Indeed, both basophil generation in the BM and basophil accumulation in the peripheral tissues are significantly impaired in mice deficient in IL-3 (Shen *et al.,* 2008). IL-3 plays an additional key role in recruiting circulating basophils into the lymphoid tissues (Kim *et al.,* 2010).

The above results strongly suggest that IL-3 plays an important role in the inducible formation of basophils. Interestingly, IL-3 does not seem to be necessary for basophil differentiation under homeostatic conditions, as IL-3-deficient mice have normal basal levels of basophils but are not able to mount a basophilia in response to parasite infections (Shen *et al.,* 2008). IL-3 also has a unique capacity to augment the proinflammatory properties and phenotype of human basophils. It has been shown that basophils rapidly bind and utilize the IL-3 they produce, as evidenced by functional and phenotypic activity that is inhibited in the presence of neutralizing anti-IL-3 receptor

(CD123) Abs (Schroeder *et al.,* 2009). *In vitro*, IL-3 also enhances acute IL-4 production in basophils (Valent *et al.,* 1989; Le Gros *et al.,* 1990). IL-3 stimulation can directly induce moderate IL-4 production in resting basophils (Hida *et al.,* 2009). In addition, IL-3 has been described to have an important priming role in antibody-dependent basophil activation and cytokine production (Le Gros *et al.,* 1990; Brunner *et al.,* 1993). Whereas IL-4 production can be detected after IgE crosslinking or protease activation of basophils, IL-3 pretreatment significantly increases IL-4 production (Hida *et al.,* 2009). Interestingly, this IL-3-mediated priming effect seems to exert an effect through a different receptor complex than that inducing IL-3-mediated basophil development (Hida *et al.,* 2009). This may be particularly relevant for basophil-T cell crosstalk *in vivo* as activated T cells produce IL-3 (Shen *et al.,* 2008). When starved of IL-3, basophils will produce small amounts of IL-4 directly in response to subsequent IL-3 stimulation. This depends on the presence of the Fc receptor common γ-chain (FcRγ), as FcRγ knockout mice are unable to produce IL-4 in response to IL-3 stimulation. The FcRγ chain interacts with the IL-3 receptor complex; IL-3R signaling through FcRγ induces IL-4 production (Hida *et al.,* 2009). FcRγ is a constitutive component of the IL-3R and is essential, through its ITAM, in the IL-3-induced production of IL-4 and in supporting Th2 differentiation by basophils. The involvement of FcRγ in IL-4 production is selective, as another function of IL-3, the promotion of basophil proliferation, was not affected by FcRγ deficiency (Hida *et al.,* 2009).

It has been suggested that basophils play important roles in the development of type 2 immunity (Mitre and Nutman, 2006). It has been demonstrated that basophils, defined as $CD49b^+$ $Fc\varepsilon RI^+$ cells, promote differentiation of activated naive CD4 T cells into IL-4-producing Th2 type effector cells by providing IL-4 necessary for Th2 differentiation (Oh *et al.,* 2007). *In vivo*, CD4 T cells primed in the presence of elevated numbers of basophils preferentially differentiate into IL-4-producing effector cells (Oh *et al.,* 2007).

Moreover, spontaneous Th2 immunity develops in mice deficient in interferon regulatory factor 2 where basophil generation is elevated (Hida *et al.,* 2005). Basophils were also shown to induce IgE-mediated chronic allergic dermatitis and allergen-induced Th2 differentiation (Sokol *et al.,* 2008). In support of this, depletion of basophils dramatically abolished the development of inflammatory lesions, reduced infiltration of other effector cells (Obata *et al.,* 2007) and abrogated the Th2 differentiation induced by allergen immunization (Sokol *et al.,* 2008). Thus, there is considerable evidence supporting the hypothesis that basophils are important immunoregulatory cells particularly in Th2 type immunity. Recent work has established important roles for basophils in initiating and augmenting Th2-type immune responses to allergen challenges and parasitic infection (Sokol *et al.,* 2008; Min and Paul, 2008).

1.4.3 GM-CSF biology

The GM-CSFRα subunit is expressed on $CD34^+$ progenitor cells and all myeloid lineages, as well as some myeloid leukemias and vascular endothelial cells (Gauvreau *et al.,* 1998). GM-CSF is secreted by activated T lymphocytes, eosinophils, mast cells, basophils, macrophages, endothelial cells and bone marrow stromal cells. In addition to the promotion of myeloid differentiation, GM-CSF is a potent inhibitor of $CD34^+$ progenitor cell differentiation into lymphoid progenitors or type 2 dendritic cells (Iwasaki-Arai *et al.,* 2003). Likewise, GM-CSF inhibits the terminal differentiation of mast cells and decreases FcεR1 expression and tryptase production by mature mast cells (Welker *et al.,* 2001). Recombinant human GM-CSF has been used clinically to promote myeloid cell recovery in myelosuppressed patients and is currently under investigation as an adjuvant for vaccines (Pichichero, 2008). The *in vitro* inhibition of

mast cell differentiation by GM-CSF also led to the use of GM-CSF as a treatment of systemic mastocytosis, although with limited benefit (Zuberbier *et al.*, 2001).

When the gene encoding βc or the GM-CSF gene was deleted in mice, these animals had normal numbers of hematopoietic stem cells and progenitor cells but failed to accelerate production of granulocytes, macrophages, and eosinophils when challenged with infectious and inflammatory stimuli (Robb *et al.*, 1995; Nishinakamura *et al.*, 1995). However, the GM-CSF null mice also revealed a totally unexpected phenotype and elucidated a new role for GM-CSF (Robb *et al.*, 1995). These mice had pulmonary alveolar proteinosis (PAP), which is characterized by abnormal surfactant metabolism. A similar PAP phenotype occurred when the gene encoding βc was deleted (Robb *et al.*, 1995; Nishinakamura *et al.*, 1995). In the GM-CSF null or βc null mice, as well as in human PAP, there is an accumulation of phospholipids and surfactant proteins within alveolar spaces. In addition, neutralizing auto-antibodies against GM-CSF were detected in adult patients with PAP (Bonfield *et al.*, 2002) and disease activity appears to correlate with the anti-GM-CSF antibody titer (Bonfield *et al.*, 2002). Very recently, it has been shown that PAP is caused by deletion of the GM-CSFR gene in the X chromosome pseudoautosomal region and new studies also identified three patients in whom PAP was caused by mutations in the gene encoding the ligand-binding α chain of the GM-CSF receptor (Notarangelo and Pessach, 2008). These data, together with those of the gene knock-out studies, strongly suggest that PAP is caused by a lack of GM-CSF function, which is likely to be important for modulating pulmonary alveolar macrophage metabolic activity.

GM-CSF has also been implicated in cellular transformation. Expression of both GM-CSFRα and βc has been detected in different types of malignancies, indicating that GM-CSF can potentially transduce proliferative and survival signals to these transformed

cells (Baldwin *et al.,* 1989). Examples of these malignancies include chronic myeloid leukemia, acute myeloid leukemia, juvenile myelomonocytic leukemia, small-cell lung carcinoma, melanoma, certain breast cancer cell lines and prostate cancer. The transient improvement of a patient with a GM-CSF responsive myelomonocytic leukemia treated with a recombinant GM-CSF that had been mutated at Glu21, to prevent βc signaling suggests the potential to treat such diseases with GM-CSF antagonists or GM-CSFRα antagonists (Bernard *et al.,* 2002). However, the effects claimed for E21R hGM-CSF could not be reproduced and the clinical trial was dropped (Jakupovic *et al.,* 2004). GM-CSF has also been shown to be involved in angiogenesis through the JAK2/STAT3 pathway *in vivo* (Valdembri *et al.,* 2002). Using chicken chorioallantoic membranes, GM-CSF stimulation was found to induce vessel sprouting from aortic rings without recruitment of inflammatory cells. Taken together, these data indicate that unregulated GM-CSF signaling can have physiological consequences leading to the promotion of malignant growth.

1.4.4 Role of IL-3, IL-5 and GM-CSF in allergic inflammation of the airways

IL-3, IL-5 and GM-CSF are related hematopoietic cytokines that are important for allergic inflammation. Allergic asthma is a chronic inflammatory disease of the airways (Braman, 2006). Inflammatory infiltrates in the asthmatic airways are complex, and a range of cells has been associated with disease progression. These include basophils, eosinophils, effector T lymphocytes, mast cells, macrophages, neutrophils, and, more recently, T regulatory and NK-T cells (Umetsu and Dekruyff, 2006). Th2 cytokines (including IL-3, IL-5 and GM-CSF) and eosinophils are thought to play critical roles in the induction of airway hyperreactivity (AHR) and the development of lesions that underpin chronic airway wall remodeling (Cohn *et al.,* 2004).

Several lines of evidence indicate that IL-5 is important in the pathophysiology of allergic inflammation and asthma (Huang *et al.,* 2003). Peripheral blood eosinophil counts increase within 24 hours of allergen challenge, and eosinophil infiltration of the lungs is a hallmark of asthma (Huang *et al.,* 2003). Furthermore, eosinophil-derived mediators are both toxic to the bronchial epithelium and can cause bronchospasm. In addition, IL-5 levels, Th2 T cells, and eosinophils are all increased in the bronchoalveolar lavage fluid of asthmatic patients, and the levels correlate with the severity of disease. Similar increases of IL-5, Th2 cells, and eosinophils are found in the bronchial mucosa of patients with severe asthma (Huang *et al.,* 2003).

IL-3 and GM-CSF, which together with IL-5 are the only known mediators capable of inducing eosinophil differentiation from bone marrow progenitors, are central to the survival, migration, and activation of this granulocyte (Lopez *et al.,* 1986; Rothenberg *et al.,* 1988). IL-3 and GM-CSF, produced by activated T cells as well as in copious amounts by lung epithelial cells in the case of GM-CSF, share a common β receptor chain (βc) with IL-5. Indeed, signaling redundancy between these cytokines may be the critical pathway that promotes the development and recruitment of eosinophils independently of IL-5 at sites of allergic inflammation. Interestingly, limited suppression of pulmonary eosinophil numbers and AHR has been documented in a rat model of allergic asthma following partial (~60%) attenuation of the βc using antisense oligonucleotides (Allakhverdi *et al.,* 2002).

IL-5-deficient ($IL\text{-}5^{-/-}$) mice have proved extremely valuable in definition of the biological roles of IL-5 and eosinophils in host defence, bronchoconstriction, and allergic inflammation (Kopf *et al.,* 1996). In IL-5-deficient mice, the eosinophilia, lung damage, and airways hyperreactivity normally resulting from aeroallergen challenge were abolished. IL-5 and eosinophils are central mediators in the pathogenesis of

allergic lung disease (Foster *et al.,* 1996). βc-deficient (βc $^{-/-}$) mice were used in an acute model of allergic airways inflammation to show that the βc receptor chain is not only essential for eosinophil accumulation in tissues, but also plays a critical role in the development of hallmark features of asthma, including production of Ag-specific IgE, mucus hypersecretion, and AHR, by limiting the functional capacity of Th2 cells in the lung. Thus, βc plays a novel role in allergic disease by regulating multiple effector arms associated with the allergic inflammatory response (Asquith *et al.,* 2008).

Signaling through the βc by IL-3 and GM-CSF is also important in the development and function of other immune cells that play central roles in allergic inflammation. This includes dendritic cells (Storozynsky *et al.,* 1999), which play an integral role in the immune response to inhaled allergens (Lambrecht *et al.,* 2000). Importantly, IL-3 is also a critical molecular switch for the differentiation, survival, and effector function of mast cells and basophils, whereas GM-CSF is a strong activator of neutrophil and macrophage function (Lantz *et al.,* 1998). Thus, the central role of the βc in regulating the function of a range of immune effector cells suggests that this molecule may be a pivotal regulator of allergic inflammation.

1.5 IL-3 and GM-CSF receptors

One of the characteristic features of cytokines is their functional pleiotropy, i.e. a particular cytokine can exhibit a wide variety of biological actions on various tissues and cells. Such functional pleiotropy and redundancy was initially thought to be a consequence of the structural characteristics of the cytokines themselves, though the critical role of cytokine receptors is now recognized. The overlapping nature of the downstream signaling proteins recruited by the receptors to elicit the cytokine stimulus can account for the functional pleiotropy and redundancy of cytokines. Most cytokine receptors consist of a multi-subunit protein complex: a unique and specific ligand

binding subunit, and a signal transducing subunit, which may be structurally similar to other members of the cytokine receptor superfamily (Hunter, 1993; Stahl and Yancopoulos, 1993; Kishimoto *et al.,* 1994; Taniguchi, 1995). The signal transducing subunit can be regarded as the 'work-horse' of the cytokine receptor complex. Not only does it receive signals initiated upon cytokine binding, but it is also responsible for propagation of the signals to downstream target proteins. The signal transducing subunit recruits cytoplasmic proteins, which results in changes in protein-protein interactions as well as changes in the phosphorylation status of certain proteins.

IL-3, IL-5 and GM-CSF receptors conform to the shared cytokine receptor model outlined above having cytokine-specific α-subunits (Gearing *et al.,* 1989; Kitamura *et al.,* 1991; Tavernier *et al.,* 1991; Murata *et al.,* 1992; Takaki *et al.,* 1993) and a shared βc-subunit (Hayashida *et al.,* 1990). Both the α- and βc-subunits are members of the class I cytokine receptor superfamily, a family of receptors characterized by ectodomains, which contain a functional unit known as the cytokine-receptor homology module (CRM). The CRM is formed by two barrel-like domains, each composed of seven β-strands, with approximate fibronectin type-III topology. The hallmark of the class I cytokine receptors' CRM is a conserved arrangement of disulfide bonds in the N-terminal fibronectin type-III domain and the conserved WSXWS motif (where, W = tryptophan, S = serine, and X = any amino acid) in the C-terminal domain. On the basis of these conserved sequence elements in receptor ectodomains, Bazan (1990) described the superfamily of class I cytokine receptors or hematopoietin receptors. Notably, the cytoplasmic domains of these receptors lack any catalytic activity (Sakamaki *et al.,* 1992), but when the cytokine ligands bind to the oligomeric receptor, the receptor subunits are orientated so that the Janus Kinases (JAKs) that are constitutively associated with the receptor cytoplasmic domains are able to transactivate one another and initiate signaling. The signaling pathways that may be initiated in this manner are

extensive and include the Ras/MAPK (mitogen-activated protein kinase), PI-3K (phosphatidylinositol-3-kinase), and JAK/STAT (signal transducers and activators of transcription) pathways (de Groot *et al.,* 1998).

There have been many models proposed for activation of class I cytokine receptors, i.e. ligand-induced receptor dimerization, ligand-induced receptor aggregation and a ligand-induced conformational change resulting in signal transduction. More recent studies using full length GHR have shown that the receptor is found as a dimer on the surface of the cell in the absence of GH demonstrated by FRET and BRET, spectral techniques which allow one to measure protein association in live cells and membranes (Gent *et al.,* 2002; Brown *et al.,* 2005). This current model of GH signaling is not unique to GHR as other recent studies have also suggested a similar activation mechanism for the close related EPOR (Seubert *et al.,* 2003; Lu *et al.,* 2006). Recent evidence indicates that surface EPOR exists as a dimer in the absence of EPO, and that EPO binding likely activates the EPOR through defined conformational changes (Constantinescu *et al.,* 2001; Seubert *et al.,* 2003). The EPOR transmembrane and juxtamembrane segments play key roles in properly orienting the EPORs in both its active liganded and inactive unliganded states, and these studies suggest an activation mechanism that might similarly be utilized by other class I cytokine receptors.

1.5.1 IL-3 receptor

IL-3 signaling in human cells involves an IL-3-specific α receptor and a β common (βc) receptor shared with IL-5 and GM-CSF (Kitamura *et al.,* 1991). Signaling through the βc receptor requires the formation of a high affinity complex involving each cytokine and its respective α subunit. Whereas the α subunits bind their ligands with low affinity, βc does not measurably bind any of the ligands alone. In mouse cells, there is a shared βc receptor but also an IL-3-specific β receptor ($\beta_{IL\text{-}3}$).

All of the biological effects of IL-3 are mediated via the IL-3 receptor, a member of the gp140 group of the type I cytokine receptor superfamily. In contrast to the homodimeric receptors of M-CSF and G-CSF and similar to the heterodimeric GM-CSF receptor discussed above, the IL-3 receptor is composed of two distinct chains, α and β. The α chain is the primary binding chain that binds IL-3 with low affinity (Kd 50–100 nM) and has rapid dissociation kinetics. The β chain, although it does not display intrinsic binding affinity for IL-3 alone, is necessary for signal transduction and mediates the high affinity binding of IL-3 together with the α receptor (Kd 100–300 pM) in a complex that displays slow dissociation kinetics (Metcalf *et al.,* 1995). $\beta_{IL\text{-}3}$ differs from the mβc subunit in its ability to bind mIL-3 directly, although the presence of the mIL-3Rα subunit is absolutely required for signaling (Hara and Miyajima, 1992; Murphy *et al.,* 2004).

1.5.1.1 IL-3 receptor α-chain

The IL-3 receptor α-subunit (CD123) is a type I transmembrane glycoprotein belonging to the cytokine receptor superfamily, as are the related IL-5Rα and GM-CSFRα to which it displays 54 and 43% sequence homology, respectively (Lindemann and Mertelsmann, 1995). The IL-3Rα gene encodes for a protein of 41.3 kDa (Moretti *et al.,* 2001). Cleavage of the 18 amino acid signal peptide yields a mature polypeptide of 360 amino acids containing a 288 amino acid extracellular domain that plays a significant role in receptor binding to IL-3, a 20 amino acid transmembrane domain, and a short cytoplasmic domain of 52 amino acids. The extracellular domain consists of an N-terminal region of about 100 amino acids that share significant sequence homology to similar regions in the GM-CSF and IL-5 receptor α-chains, followed by a cytokine receptor domain containing four conserved cysteine residues and a WSXWS motif common to other members of this receptor family (Nicola, 1991). The extracellular

domain contains six potential N-glycosylation sites, and the presence of N-linked oligosaccharides is essential to both ligand binding by the α-subunit as well as signaling by the IL-3 receptor (Yang, 1997). N-glycosylation at these sites appears responsible for the observed molecular weight of 70 kDa. The cytoplasmic domain contains a short proline-rich motif similar to Box 1 of the common β-chain, but displays no intrinsic enzymatic activity.

The role of IL-3 in the promotion of hematopoietic progenitor cell survival and proliferation is well documented. However, the role of IL-3 in differentiation of hematopoietic cells is not very clear. Evans *et al.,* (1999) transfected the murine multipotential hematopoietic progenitor cell line FDCP-mix with chimeric receptor subunits consisting of the extracellular domain of the IL-3Rα and the intracellular domain of the GM-CSFRα subunit. This chimeric receptor promoted differentiation in response to IL-3, demonstrating the role of the α subunit in conferring specificity and determining cell fate via distinct signal transduction events. The IL-3Rα subunit (CD123) is overexpressed on leukemic stem cells and blasts suggesting a role in leukemia pathogenesis (Jordan *et al.,* 2000; Munoz *et al.,* 2001; Testa *et al.,* 2002). The increased expression of IL-3Rα is associated with enhanced blast proliferation, increased cellularity, and a poor prognosis (Testa *et al.,* 2002).

Our group recently identified a new alternatively spliced isoform of the IL-3Rα receptor in mouse (GI: 5599968; from a placental library) and human (GI: 19018027; from an RPE and choroids library) cells amongst the mRNA sequences in the NCBI EST databases. The new isoform (designated SP2) is depicted in Fig.1.2 (Chen *et al.,* 2009) and lacks domain 1 of the extracellular portion of the original IL-3Rα receptor (designated IL-3Rα SP1). The ability of the IL-3Rα SP2 and SP1 to promote differentiation of the multi-potential hematopoietic cell line FDCP-mix and the mouse

myeloid leukemia line M1 has been investigated in our laboratory. In this study, the human IL-3Rα SP2 together with hβc was shown to promote the differentiation of multi-potential FDCP-mix cells into basophil-like cells in contrast to the IL-3Rα SP1 isoform which promoted self-renewal. Human IL-3Rα SP2 also directed the differentiation of the myeloblastic M1 leukemia line into monocytes whereas IL-3Rα SP1 did not (Chen *et al.*, 2009). The ability of the two structural isoforms of the IL-3α receptor to achieve different signaling outcomes with respect to differentiation and self-renewal indicates greater complexity in the modes of receptor activation and signaling than previously recognized. More recently, our group has obtained evidence that the mIL-3Rα SP2 isoform plays an important role, not only in the formation of basophils from bone marrow progenitors in mice, but also in their maturation and ability to produce IL-4 after IL-3 stimulation (J. Chen and I. Young, unpublished).

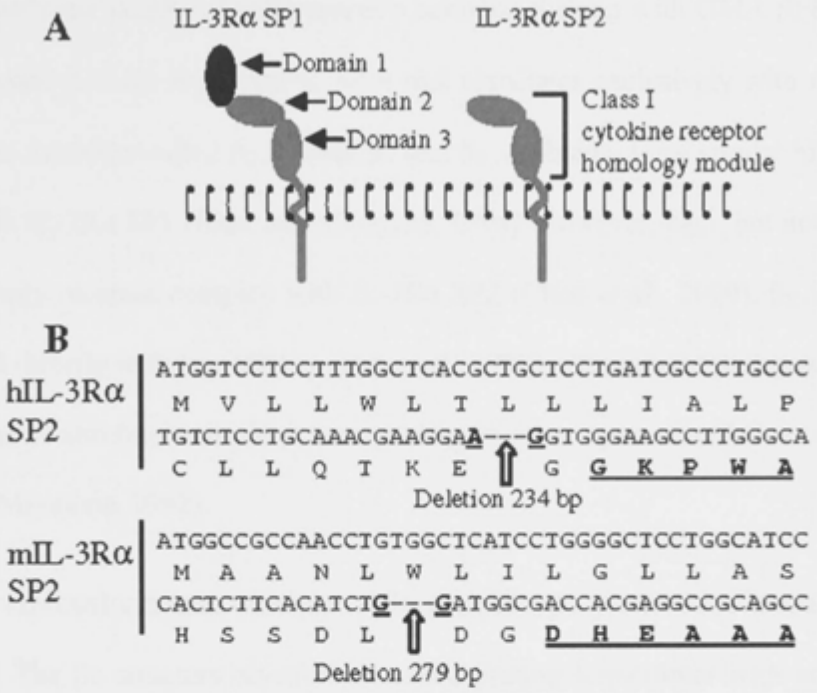


Figure 1.2: Structure of the SP2 isoform of mouse and human IL-3Rα (adapted from Chen *et al.,* 2009). A, Schematic models of IL-3Rα SP1 and SP2 isoforms showing the structural domains; B, DNA and protein sequence covering the N-terminal region of human and mouse IL-3Rα SP2 showing the position of the deletion of exons 3 and 4, which encode domain 1 of the extracellular region (indicated by the arrows). The start of the mature protein sequence (predicted by SignalP 3.0) is shown bold and underlined. The full-length nucleotide sequences for the SP2 isoforms are found in GenBank™ with accession numbers of FJ550346 for mouse IL-3Rα SP2 and FJ550347 for human IL-3Rα SP2.

1.5.1.2 IL-3 receptor β chain

In human cells the IL-3Rα subunit shares a common β chain with GM-CSFRα and IL-5Rα. A second β chain is present in mice that associates exclusively with the IL-3Rα chain and is therefore called $\beta_{IL\text{-}3}$. Both βc and $\beta_{IL\text{-}3}$ subunits form similar high-affinity IL-3Rs with IL-3Rα SP1 (Hara and Miyajima, 1994). However, $\beta_{IL\text{-}3}$, but not βc, forms a high affinity receptor complex with IL-3Rα SP2 (Chen *et al.,* 2009). $\beta_{IL\text{-}3}$ is able to bind mIL-3 directly with low affinity (Itoh *et al.,* 1990), although the presence of the IL-3Rα subunit is absolutely required for signaling to occur upon stimulation with mIL-3 (Hara and Miyajima, 1992).

Our group reported a crystal structure of the complete βc extracellular domain (Carr *et al.*, 2001). The βc structure revealed an interdigitating homodimer with an arch-like conformation (Fig. 1.3). The βc interlocking homodimer structure is also distinct from that of monomeric cytokine receptors like GHR and EPOR. Because the cytoplasmic domains of the homodimeric βc are too far apart to activate each other for signaling, βc activation would require proximate association with a ligand- bound IL-3Rα, IL-5Rα, or GM-CSFRα (Carr *et al*, 2001; Murphy and Young, 2006). The crystal structure of βc, together with mutagenesis studies (Murphy *et al.,* 2004), led to the proposition that the cytokine-binding site is composed of D1 of one chain and D4 of the partner chain in the βc homodimer that join in an antiparallel fashion.

Hansen *et al.,* (2008) reported the crystal structure of the human GM-CSF/GM-CSF receptor ternary complex and its assembly into an unexpected dodecamer or higher-order complex. These authors showed through mutagenesis of the GM-CSF receptor at the dodecamer interface that dodecamer formation is required for receptor activation and signaling. It was proposed that this unusual form of receptor assembly likely applies

also to IL-3 and IL-5 receptors, providing a structural basis for understanding their mechanism of activation and for the development of therapeutics (Hansen *et al.,* 2008).

Recently our group reported the identification of residues clustering around the highly conserved A-helix residue, Glu (23), in the mIL-3 A- and C-helices as critical for receptor binding and growth stimulation via the β_{IL-3} and mIL-3Rα SP2 subunits, whereas an overlapping cluster was required for binding and activation of β_{IL-3} in the presence of mIL-3Rα SP1. Similarly, our studies of human IL-3 indicate that two different modes of βc binding are utilized in the presence of the hIL-3Rα SP1 or SP2 isoforms, suggesting a possible conserved mechanism by which the relative orientations of receptor subunits are modulated to achieve distinct signaling outcomes (Mirza *et al.,* 2010a). Our group also described a role for the domain 1 D-E loop disulfide of hβc and β_{IL-3} in maintaining the precise positions of ligand-binding residues necessary for normal high affinity binding and signaling (Mirza *et al.,* 2010b).

Structure/function studies using the common β-chain of IL-3 have shown that the cytoplasmic domain of this chain can be divided into two distinctive functional domains (Woodcock *et al.,* 1999). The membrane proximal intracellular portion of the receptor till amino-acid number 589, that includes the Box 1 region, was shown to be necessary and sufficient for DNA synthesis mediated by several of these cytokines including IL-3 (Tian *et al.,* 1996). This region was also found to be required for the induction of proliferation-associated genes such as c-myc, pim-1 and oncostatin M. The membrane distal domain of the receptor, spanning amino acids 589–881, was shown to be required for growth inhibition that can be induced by some of the cytokines. More importantly, this region appears to be necessary for the promotion of viability of hematopoietic cells (Inhorn *et al.,* 1995).

IL-3, GM-CSF, and IL-5 share a common β subunit for the formation of their high affinity receptors and subsequent activation of downstream signaling pathways (Martinez-Moczygemba and Huston, 2003). Generally, these receptors are differentially expressed among cells of the hematopoietic system. However, cross-competition between the cytokine ligands for the βc chains results in the formation of a hierarchy of βc binding in those cells expressing the appropriate α chains as well as the βc chain (Lopez *et al.,* 1991; Lopez *et al.,* 1992). GM-CSF shows the greatest ability to recruit βc, followed by IL-3 and IL-5. As such, affinity for the βc subunit may induce differential biological activities even in the presence of more that one of the cytokine ligands and/or their specific α-chains.

β chains are widely expressed throughout the hematopoietic system. Despite them being able to associate with an array of adaptors and signaling molecules, it is important to recognize that the patterns of expression of these proteins differs depending on the cell type, maturation stage, and the proliferation and/or activation signals that the cell is exposed to. As such, the end-result is intimately dependent not only on a functional receptor that can transduce extracellular signals, but also in the intracellular environment that must provide the necessary cytoplasmic signaling proteins to generate a downstream response.

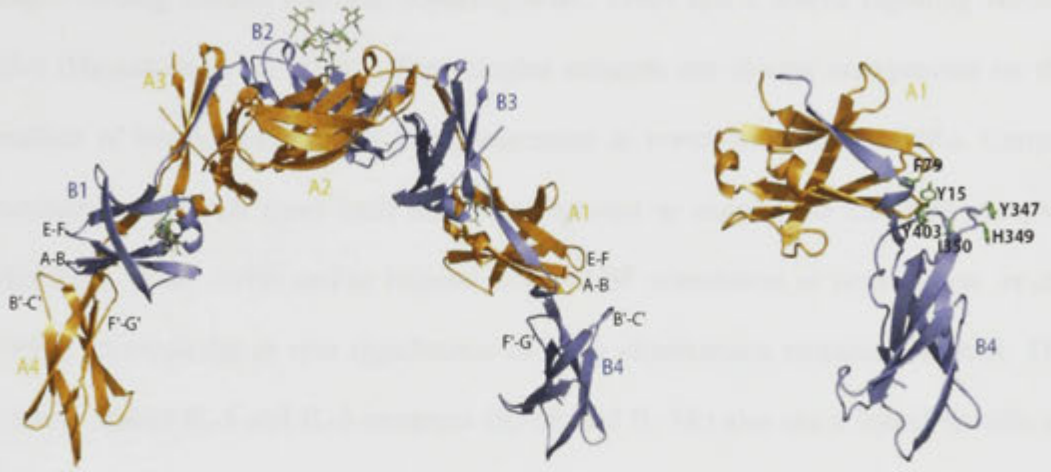


Figure 1.3: The structure of the human βc ectodomain (adapted from Carr *et al.*, 2001). Left panel: the intertwined chains of the βc homodimer are colored orange (''A'' chain) and blue (''B'' chain) and the component domains of each chain are labeled in orange or blue text, respectively. N-linked glycosylation chains are shown as sticks at N34 and N167 (carbons are colored green; nitrogens, blue; and oxygens are colored red). The loops that contribute residues to the functional epitope for IL-3, IL-5, and GM-CSF binding are labeled in black text. Right panel: expanded view of the domain B1-A4 interface. The color scheme is as for the left panel, except the side chains of the functional epitopes residues are shown in green and are labeled with black text. The coordinates for βc are available from the Protein Data Bank (accession 1GH7).

1.5.2 GM-CSF receptor

The GM-CSF receptor, first identified on cells of the myelomonocytic lineage by ligand-binding studies (Walker and Burgess, 1985), is a heterodimer that comprises a major binding subunit (GMRα) (Gearing *et al.,* 1989) and a shared signaling subunit (βc) (Hayashida *et al.,* 1990). The receptor subunits are always coexpressed on the surface of leukocytes, with βc being expressed at lower levels than GMRα. Certain nonhemopoietic cell types have also been reported to express the GM-CSF receptor (Baldwin *et al.,* 1989) and to respond to GM-CSF stimulation *in vitro* (Rivas *et al.,* 1998), although the *in vivo* significance of these observations remains uncertain. The closely related IL-3 and IL-5 receptors (IL-3R and IL-5R) also use a ligand-specific α-chain and share βc with the GM-CSF receptor. Although βc is absolutely required for signaling in cells expressing GM-CSF, IL-3, or IL-5 receptors, there is evidence that the functional specificity of signaling may be fine-tuned by the presence of different α-chains. The GM-CSF receptor can signal an astonishing variety of cellular functions, including protection from apoptosis, entry and progression through the cell cycle, early commitment to myelopoiesis, differentiation/maturation of committed progenitors, and multiple activation and motility functions in mature cells (Metcalf, 2008).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and the related cytokines IL–3 and IL-5 regulate the production and functional activation of hematopoietic cells, with GM-CSF acting on monocyte/macrophages and all granulocytes (Metcalf, 2008). GM-CSF also controls dendritic cell and T-cell function (Mellman and Steinman, 2001), thus linking innate and acquired immunity. Because of the widespread expression of the GM-CSF receptor in hematopoietic cells, it was assumed that both GM-CSF and its receptor were key players in the regulation of steady-state functions. Whereas this turned out to be true in terms of lung physiology (Dranoff *et al.,* 1994),

deletion of either the GM-CSF gene or its receptor showed no obvious deficiency in myeloid cell numbers or production. Rather, a growing body of evidence now suggests that GM-CSF plays a key role in signaling emergency hematopoiesis (predominantly myelopoiesis) in response to infection, including the production of granulocytes and macrophages in the bone marrow and their maintenance, survival, and functional activation at sites of injury or insult (Hamilton and Anderson, 2004; Fleetwood *et al.*, 2005).

1.6 IL-3 induced signal transduction pathways

IL-3 induces three types of biological effects in target cells, consisting of the induction of survival, proliferation and cell differentiation. These different biological effects require the activation of several signal transduction pathways, resulting in the reprogramming of gene expression. The first event in the mechanism of induction of a cell response to IL-3 is represented by the binding of this cytokine to its specific membrane receptor: the initial binding of IL-3 to the IL-3Rα chain results in the recruitment of a β-chain homodimer with the consequent formation of a high-affinity heterodimeric complex, culminating in initiation of cell signaling. The intracellular cell signaling originated by activation of the IL-3R involves three main known signaling transduction pathways: the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathways and the phosphatidylinositol 3-kinase (PI3-kinase) pathway (Fig. 1.4).

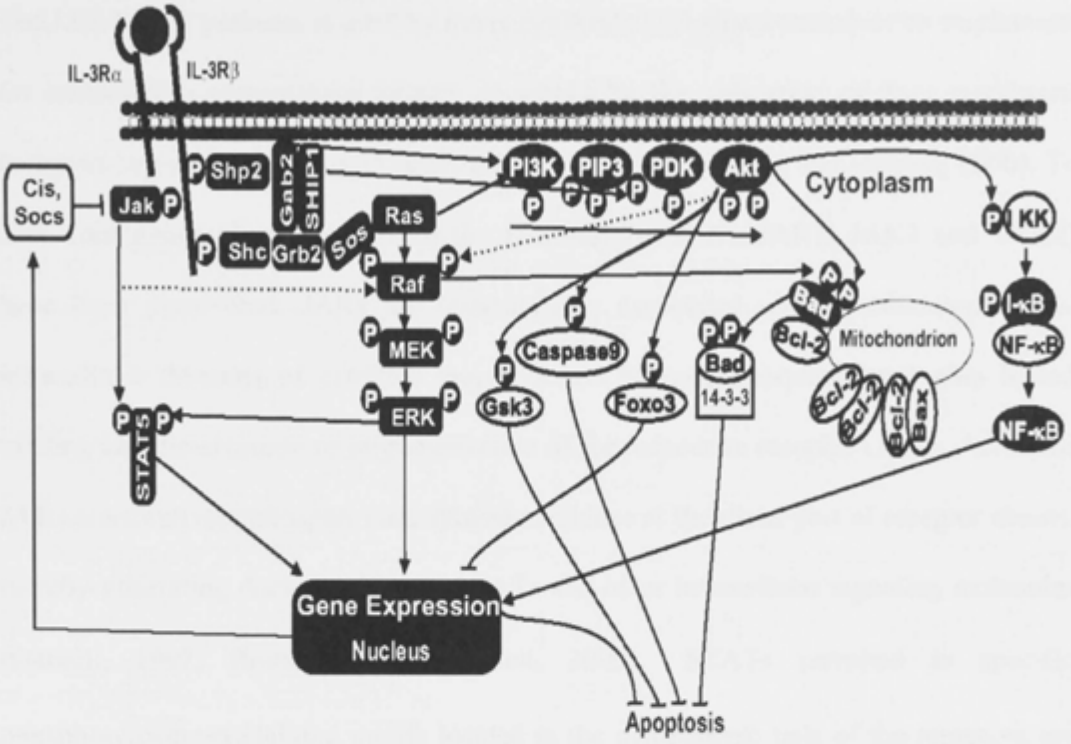


Figure 1.4: IL-3 signaling (from Steelman *et al.,* 2004). IL-3 receptor activation induces the JAK/STAT, Raf/MEK/ERK and PI3K/Akt pathways. The JAK/STAT pathway induces the expression of anti-apoptotic genes such as Bcl-XL and also CIS and SOCS proteins, which serve as negative feedback regulators. The Ras/Raf/MEK/ERK pathway phosphorylates transcription factors that promote cell proliferation. Akt can phosphorylate many targets (Bad, $I^{\kappa}K$, caspase-9, GSK3β, Foxo3), which have effects on apoptosis and cell cycle progression. IL-3 also induces tyrosine phosphorylation of Gab2, a docking molecule, which associates with the Shp-2 phosphatase, Grb-2 and PI3PI3K. Gab2 can regulate PI3K and Ras activities. IL-3 regulates expression of phosphatases such as Shp2 and SHIP1, which regulate these pathways. Dotted lines indicate potential interactions between the pathways. Solid lines indicate direct paths of signal transduction.

1.6.1 JAK/STAT pathway

The JAK/STAT pathway is used by many cytokines as a signal transduction mechanism for transmitting extracellular signals generated by the activation of their membrane receptors to promoters of target genes (Darnell, 1997; Bromberg and Darnell, 2000). To date, four mammalian members of the JAK family (JAK1, JAK2, JAK3 and TYK2) have been discovered. JAKs are constitutively associated with membrane-proximal intracellular domains of cytokine receptors and become phosphorylated after ligand binding and dimerization or oligomerization of the respective receptor chains. Activated JAKs constitutively phosphorylate tyrosine residues at the distal part of receptor chains, thereby generating docking sites for STATs and other intracellular signaling molecules (Darnell, 1997; Bromberg and Darnell, 2000). STATs recruited to specific phosphotyrosine-containing motifs located in the cytoplasmic tails of the receptors are in turn phosphorylated by activated JAKs at distinct tyrosine residues. After this modification, STATs either homodimerize or heterodimerize, leave the receptor complex, enter the nucleus and bind to response elements of target genes, thus influencing their transcriptional activity (Darnell, 1997; Bromberg and Darnell, 2000). Gene targeting experiments have provided clear evidence that JAK2 is strictly required for both the development of the hematopoietic system (Neubauer *et al.,* 1998) and for the response to cytokines, including IL-3 (Parganas *et al.,* 1998).

Although JAK2 is the kinase predominantly activated by IL-3, members of the Src family of kinases, such as Fyn, Lyn, Syk, Btk and Hck, have also been reported to be activated by IL-3. JAK activation results in the phosphorylation of the β-chain at six tyrosine residues: Tyr^{577}, Tyr^{612}, Tyr^{695}, Tyr^{750}, Tyr^{806} and Tyr^{866} (Geijsen *et al.,* 2001). Three of these tyrosine residues, Tyr612, Tyr695 and Tyr750, serve as docking

sites for the Src homology 2 (SH2) domains of two members of the STAT family: STAT1 and STAT5. These two STATs are recruited to the receptor complex by their SH2 domains, become tyrosine-phosphorylated on critical residues in their carboxytermini and then homodimerize or heterodimerize by means of reciprocal SH2 and phosphotyrosine interactions.

The two STATs that play the most relevant role in mediating the biological effects of IL-3/IL-3R are STAT5 and STAT3. As discussed above, STAT5 activation is dependent on the β-chain, while STAT3 activation is mainly dependent on the cytoplasmic tail of the IL-3Rα-chain (Piu *et al.,* 2002). The target genes of STAT3 and STAT5 are represented by a set of genes involved in survival, proliferation and differentiation. In this complex, a remarkable activating effect is exerted by STAT5 on retinoic acid receptors (RARs). These receptors regulate the growth and differentiation of primitive myeloid precursors *in vitro* and knockout mice deficient in RARα/RARγ display a block in granulocytic differentiation (Labrecque *et al.,* 1998). IL-3 markedly stimulates RAR expression through a STAT5-dependent mechanism; interestingly, activated STAT5 and RARs physically interact, forming a heterodimer (Si and Collins, 2002)

1.6.2 MAPK Pathways

The MAPK pathways include three main signaling cascades: the extracellular signal-regulated kinase (ERK), c-jun amino-terminal kinase (JNK) and p38. These pathways are key mediators of a large number of important cellular responses that play a major role in the control of cell survival and proliferation. These important signaling pathways are activated in response to many cytokines and growth factors. All three signaling pathways are activated by IL-3.

JNK is a member of the MAPK family and was identified as a stress-activated protein kinase that phosphorylates c-jun on two sites in the NH_2-terminal activation domain. The upstream pathway leading to JNK activation is complex and involves as an end step the dual phosphorylation of the motif Thr-Pro-Tyr located in the activation loop (Manning and Davis, 2003). JNK phosphorylation is mediated by two MAPKs, MAP2K4 and MAP2K7, that cooperatively activate JNK. JNK activation leads to the phosphorylation of a number of transcription factors, most notably the c-jun component of AP-1 and cellular proteins, mainly those associated with the control of cell apoptosis. This last mechanism, in particular, is responsible for the anti-apoptotic mechanism induced by IL-3 via JNK activation. In fact, JNK is responsible for the phosphorylation and consequent inactivation of the proapoptotic Bcl-2 family protein Bad; phosphorylated Bad becomes unable to bind the anti-apoptotic molecule Bcl-X_L (Yu *et al.,* 2004). IL-3 withdrawal is associated with inactivation of JNK, which leads to dephosphorylation of Bad, which determines its potent activity (Yu *et al.,* 2004).

The activation of the ERK pathway by IL-3 starts with the phosphorylation of the β-chain at the level of residue Tyr^{577}, induced by JAK2 activation. This tyrosine residue, once phosphorylated, becomes a docking site for the cellular substrate SHC (Src homology and collagen), which then in turn becomes phosphorylated, allowing it to interact with the SH2 domain of the growth factor receptor-bound adaptor protein Grb2 and the nucleotide exchange factor SOS (Ravichandran, 2001). The SHC/Grb2 interaction leads to an association with the nucleotide exchange factor for Ras, which in turn leads to the sequential activation of Ras, Raf-1, MEK and ERK. Using β-chain mutants in which a single tyrosine residue is left intact. It was shown that either Tyr^{577}, Tyr^{612} or Tyr^{695} was sufficient to promote SHC phosphorylation, Grb2 association and MAPK activation (Kinoshita *et al.,* 1995).

The downstream targets of activated ERK are represented by genes involved in the control of apoptosis and transcription factors that control the expression of genes involved in regulating proliferation. Concerning apoptosis genes, IL-3 is a stimulator of Bcl-2 and Bcl-X_L expression through an ERK-dependent mechanism (Kinoshita *et al.*, 1995). In addition to the upregulation of anti-apoptotic factors, the ERK pathway also inhibits apoptosis through inactivation of pro-apoptotic factors, such as Bad, via phosphorylation. Concerning the stimulatory effect on cell proliferation, the ERK pathway induces the transcription factors activating transcription factor 2 (ATF2) and ternary complex factor (TCF), which are capable of activating the proliferation- related genes c-Fos and c-Jun (Perkins *et al.*, 1996).

The tyrosine phosphatase Src homology protein tyrosine phosphatase 2 (SHP2) appears to be a positive regulator of the cell signaling originated by the common β-chain. In fact, the catalytic activity of SHP2 is strictly required for both JAK2 and ERK activation, but not for PI3-kinase induction (Yu *et al.*, 2003).

The activation of the MAPK p38 pathway by IL-3 is related to the induction of anti-apoptotic mechanisms. In fact, the antiapoptotic mcl-1 is an immediate-early gene activated by IL-3 through a transcriptional mechanism involving the phosphorylation of PU.1 and subsequent transcriptional activation of the mcl-1 gene by this phosphorylated transcription factor (Wang *et al.*, 2003).

1.6.3 PI3-Kinase Pathway

While β-chain tyrosine phosphorylation is required for the activation of STAT and MAPK signaling cascades, PI3-kinase activation requires phosphorylation of the β-chain at the level of Ser^{585}. The phosphorylation of Ser^{585} results in recruitment of the

adaptor molecule 14-3-3 (Stomski *et al.*, 1999). Mutation at this amino acid position causes a block in PI3-kinase activation by IL-3 (Guthridge *et al.*, 2000).

PI3-kinase is a heterodimeric lipid kinase composed of a regulatory subunit (p85) and a catalytic subunit (p110). Following IL-3 stimulation, activated PI3-kinase phosphorylates phosphatidylinositol lipids at the level of the inositol residues present in the cell membrane, thereby allowing the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3), a second messenger that interacts with proteins through pleckstrin homology domains and induces a variety of biological effects (Cartley, 2002).

One important target of PIP_3 is the serine/threonine kinase Akt. This kinase plays a key role in the survival signals originating from PI3-kinase activation. Thus, the activation of Akt in response to IL-3 produces phosphorylation of Bad, resulting in 14-3-3 binding and sequestration in the cytoplasm, with consequent cell survival (Zha *et al.*, 1996). In the absence of IL-3, PI3-kinase and Akt are not activated and Bad remains unphosphorylated, allowing it to translocate to mitochondria where it can bind the antiapoptotic proteins Bcl-2 and Bcl-X_L and induce cell death.

More recently, it was observed that Akt could promote cell survival by acting as an inducer of nuclear factor-κB (NF-κB). Under normal conditions, NF-κB is maintained in an inactive, latent state in the cytoplasm by the inhibitor protein IκB. Akt phosphorylates IκB kinase (IKK), which in turn phosphorylates IκB (Ramaskova and Makorov, 1999). Phosphorylated IκB is then targeted for degradation by the proteosome, allowing NF-κB to translocate to the nucleus and regulate gene expression. NF-κB is able to regulate the expression of a large set of genes, particularly the antiapoptotic genes Bcl-2, Bcl-XL and A1. Recent studies provided clear evidence that this pathway is involved in the stimulatory effect exerted by IL-3 on Bcl-2 gene expression (Guthridge *et al.*, 2004).

The PI3-kinase pathway is involved not only in mediating the antiapoptotic effects of IL-3, but also in the control of cell proliferation by IL-3 via induction of the c-Myc protein.

1.6.4 Pim-2 gene

Anti-apoptotic mechanisms are induced by IL-3 not only via signal transduction pathways, but also via transcriptional stimulation. This is the case for the Pim-2 protein, a member of a family of serine/threonine kinases consisting of three members (Pim-1, Pim-2 and Pim-3), the expression of which is markedly stimulated by IL-3. In particular, in an IL-3-dependent lymphoid cell line, it was shown that Pim-2 gene expression was downregulated to the greatest extent following IL-3 deprivation. The activity of Pim-2 as an anti-apoptotic factor appears to be related to phosphorylation and consequent inactivation of the pro-apoptotic proteins Bad and Bim (Fox *et al.,* 2003). The effects of Pim-2 on cell survival are similar to those displayed by Akt; however, Pim-2 does not appear to act through Akt.

1.6.5 Negative control of IL-3R signaling

Excessive uncontrolled signaling through IL-3R, as well as through other cytokine receptors involved in the stimulation of cell proliferation, could have deleterious effects and lead to uncontrolled cell growth. Therefore, several biochemical mechanisms limit in both extent and time the signaling originating from activation of IL-3R.

One mechanism is based on the activity of the suppressors of cytokine signaling (SOCS) family of small SH2-containing proteins. These proteins inhibit STAT signaling through two different mechanisms: one related to inactivation of JAK2 by blocking the access of STAT5 to receptor binding sites (Endo *et al.,* 1997), and the other to ubiquitination of JAK2 with subsequent degradation (Ungureanu *et al.,* 2002).

The inhibitory effect of SOCS-1 on STAT5 activity is potentiated by Pim-1, a serine/threonine kinase induced by IL-3. Pim-1 acts by phosphorylating SOCS-1, an event that stabilizes the interaction of this protein with STAT5 (Peltola *et al.,* 2004). It is therefore evident that both Pim-1 and SOCS-1 are components of a negative feedback mechanism that allows STAT5 to attenuate its own activity.

A second mechanism of attenuation of the STAT signaling originating from activation of IL-3R is related to the activation of cytosolic tyrosine phosphatases that control the level of phosphorylation of cellular substrates of activated tyrosine kinases. The tyrosine phosphatase SHP1 is involved in the negative control of IL-3R signaling, as shown by the observation that its overexpression resulted in a complete loss of IL-3 induced effects. SHP1 interacts directly with the IL-3R common β-chain at the level of Tyr^{612}, after phosphorylation (Bone *et al.,* 1997).

A third mechanism common to several membrane receptors is represented by receptor internalization, with subsequent degradation. In fact, for the majority of membrane receptors, including the IL-3R, ligand binding promotes the internalization of the receptor/ligand complex into clathrin-coated vesicles. These vesicles then fuse with early endosomes, where receptors are either resorted for receptor recycling back to the cell membrane or are targeted into late endosomes and finally degraded in lysosomes. Two lines of evidence suggest that this mechanism plays a role in the termination and modulation of IL-3R signaling: 1) prolonged IL-3R signaling is observed in the presence of proteosome inhibitors (Callus and Mathey-Prevot, 1998); and 2) biochemical studies indicate that during IL-3R internalization, the IL-3R common β-chain, but not the IL-3R α-chain, is proteolytically degraded at the level of the cytoplasmic domain (Martinez-Moczygamba and Huston, 2001).

1.7 Role of transcription factors and microRNAs in regulating differentiation

HSCs (hematopoietic stem cells) reside in the bone marrow and give rise to progenitors that become progressively restricted to several or single blood cell lineages (Iwasaky and Akashy, 2007). These progenitors are devoted to the production of mature blood cells. All the non-lymphoid lineages of the blood are indicated as myeloid lineages. However, in its most common usage 'myeloid cells' refers to granulocytes (neutrophils) and monocytes/macrophages. Granulocytes (neutrophils) and monocytes/macrophages develop by progenitors that include CMPs (common myeloid progenitors) and, subsequently, GMPs (granulocyte/macrophage progenitors) in a process known as myelopoiesis. The production of these two alternative lineages depends on the unique combination and relative expression levels of key transcription factors that promote competing specific genetic programmes (Rosenbauer and Tenen, 2007). In particular, the co-ordinated development of myeloid cells depends on reciprocal inhibitory effects and adjustment of the concentration of key transcription factors that are responsible for the reduction in cell proliferation, induction of apoptosis and the expression of lineage-specific myeloid genes. Among master transcription factors for myelopoiesis are C/EBPs (CCAAT/enhancer-binding proteins), PU.1, GFI1 (growth-factor independent 1), IRFs (interferon-regulatory factors), SCL (stem-cell leukemia factor; also known as TAL1) and RUNX1 [runt-related transcription factor 1; also known as AML1 (acute myeloid leukemia 1)]. A remarkable feature of the transcription factors in the myeloid system is that in human AML (acute myeloid leukemia) the majorities of them are involved in chromosomal translocations or contain somatic mutations (Tenen, 2003; Rosenbauer and Tenen, 2007).

miRNAs (microRNAs) provide an additional level of control beyond the transcription factors. They modulate a variety of developmental and physiological processes by regulating translation and stability of specific mRNAs (Bartel, 2004). A single miRNA can control the levels of hundreds of different target genes. Beyond its mere presence or absence, the concentration at which a given factor is expressed may influence lineage choice and differentiation. For this reason, it has been hypothesized that miRNAs deeply contribute to the large network of factors that dictate hematopoietic cell lineage specification. Indeed, several miRNAs are highly expressed in specific hematopoietic lineages and manipulation of their levels has been correlated with changes in cellular properties or differentiation (Chen *et al.,* 2004; Shivdasani, 2006).

1.8 Myeloid differentiation pathways

Four myeloid differentiation pathways have been recognized: (1) steady state myeloid differentiation, which maintains the normal levels of mature myeloid cells, (2) cytokine-driven (inducible or emergency) myelopoiesis which generates additional myeloid effector cells during infections or allergic responses, (3) ATRA-induced differentiation of PML cells and (4) CD44-driven differentiation of AML blasts.

1.8.1 Steady state myeloid differentiation

Studies of steady state myeloid differentiation have mainly focussed on granulopoiesis and particularly on the differentiation of neutrophils. C/EBPα is a master myeloid transcription factor and absolutely required for neutrophil differentiation (Rosenbauer and Tenen, 2007). The activity or expression of C/EBPα is commonly affected in AML (Mueller and Pabst, 2006). C/EBPα $^{-/-}$ mice are blocked in the formation of GM progenitors and C/EBPα does not appear to be required for later steps in neutrophil differentiation (Zhang *et al.,* 1997). Although most attention has been given to the role

of C/EBPα in granulocyte differentiation, fetal liver from C/EBPα $^{-/-}$ mice lacks mature macrophages and macrophage progenitors (Heath *et al.,* 2004). Progenitors from C/EBPα $^{-/-}$ mice are still able to differentiate in response to IL-3 and GM-CSF (Zhang *et al.,* 2002) indicating the existence of a cytokine-induced differentiation pathway in the progenitors which is not affected by the absence of C/EBPα. When C/EBPα is introduced into the C/EBPα $^{-/-}$ progenitors, the G-CSFR, C/EBPβ, C/EBPε and miR-223 are upregulated and the cells differentiate into neutrophils (Zhang *et al.,* 2002).

1.8.2 Cytokine-induced myeloid differentiation

Experiments with C/EBPα $^{-/-}$ progenitors indicate that IL-3 and GM-CSF induce differentiation of these cells to neutrophils and upregulate C/EBPβ and C/EBPε (Zhang *et al.,* 2002). ATRA does not induce the differentiation of C/EBPα $^{-/-}$ progenitors. Evidence has also been presented that C/EBPβ is important for cytokine-induced emergency granulopoiesis (Hirai *et al.,* 2006). IL-3- and GM-CSF-driven myeloid differentiation appears commonly to be blocked in AML. However, at the commencement of the work described in this thesis there was little known of the mechanisms regulating IL-3- and GM-CSF-driven differentiation.

1.8.3 ATRA-induced differentiation of promyelocytic leukemia cells

Acute promyelocytic leukemia has been characterized as a differentiation arrest at the promyelocyte stage due to t (15; 17) reciprocal chromosomal translocation that generates a PML-retinoic acid (RA) receptor α (RARα) fusion protein (de The *et al.,* 1991; Kakizuka *et al.,* 1991). *All-trans* retinoic acid (ATRA) induces the differentiation and elimination of APL clones (Yamanaka *et al.,* 1997). The NB4 cell line was derived from a promyelocytic leukemia patient and carries the t (15; 17) translocation resulting in the joining of the promyelocytic leukemia-associated protein (PML) oncogene to the

gene encoding the RARα (Lanotte *et al.,* 1991). Previous studies have demonstrated that NB4 cell line can be differentiated into granulocytes by ATRA (Tohda *et al.,* 1992; Clark *et al.,* 2004). Induction of differentiation by ATRA results in upregulation of the G-CSFR, C/EBPβ, C/EBPε and miR-223 (Park *et al.,* 1999; Duprez *et al.,* 2001; Fatica *et al.,* 2008). Interestingly, NB4 does not differentiate in response to IL-3 or GM-CSF. However, it has been shown that the combined neutralization of hIL-3, hGM-CSF and hG-CSF inhibits ATRA-induced differentiation of NB4 (Matsui *et al.,* 2005) suggesting a possible involvement of the hβc receptor system in the process.

1.8.4 CD44-induced differentiation of AML blasts and NB4

Differentiation of a variety of sub-types of AML cells can be induced by antibody ligation of the cell surface receptor CD44 (Charrad *et al.,* 1999). Indeed, CD44 ligation is even more efficient than ATRA in lowering the levels of the PML-RARα protein in AML3 blasts (Charrad *et al.,* 1999). Although the efficiency of differentiation varies with the AML sub-type, successful differentiation of AML primary cells or AML cell lines using anti-CD44 monoclonal antibodies has raised the exciting prospect of CD44 as a new target for anti-leukemia differentiation therapy. The anti-CD44 monoclonal antibodies of H90, A3D8 and HI44a can induce differentiation and apoptosis of human acute myeloid leukemia cells (Charrad *et al.,* 2002; Song *et al.,* 2004), Further, H90 has the capacity to eradicate human AML stem cells (Jin *et al.,* 2006). Little is currently known of the mechanisms in myeloid differentiation induced via CD44, but induction of expression of G-CSF and M-CSF mRNAs has been detected in some cases (Charrad *et al.,* 1999). It has also been shown that autocrine/paracrine secretion of GM-CSF is required for AML5 blast differentiation by ligated CD44 (Delaunay *et al.,* 2008) suggesting the involvement of the βc receptor system.

Work presented in the thesis

The promyelocytic leukemia line NB4 is not only blocked in steady-state granulocytic differentiation but also in cytokine-induced differentiation driven by IL-3 or GM-CSF. In Chapter 3, the hIL-3R system is investigated in NB4 cells. The IL-3R system was present at low levels in NB4 but was otherwise normal. Elevation of hIL-3R levels by ectopic expression restored IL-3-induced differentiation, in the absence of ATRA, but differentiation was only partial indicating that the PML-RARα protein also interferes with the induction of IL-3-driven differentiation. SiRNA knockdown of hβc did not affect ATRA-driven differentiation. In Chapter 4, the expression of CD44 and its ligand osteopontin in differentiating myeloid cells was investigated. Although it is known that CD44 is a potent inducer of differentiation of promyelocytic leukemia cells, siRNA knockdown of CD44 did not affect ATRA-induced differentiation of NB4 cells and also did not affect IL-3-induced differentiation. A major goal of the thesis work was the establishment of a new *in vitro* model for mIL-3-induced myeloid differentiation which would enable detailed molecular studies of the mechanisms involved with the potential for further investigations to be carried out *in vivo*. This work is addressed in Chapter 5. The recent development of conditional Hoxb8 (ER-Hoxb8) provides a way of reversibly immortalizing early myeloid progenitors. The activity of Hoxb8 is controlled by the level of estrogen in the medium. Growth factor-dependent immortalized myeloid progenitors can be isolated as continuous cell lines which grow without differentiation in the presence of estrogen and differentiate in its absence (Wang *et al.*, 2006). Thus ER-Hoxb8 potentially provides an excellent model for study of the mechanisms regulating the switch between growth and differentiation. It is shown in Chapter 5 that the SCF-dependent line SCF ER-Hoxb8 shows IL-3-driven differentiation when grown in the absence of estrogen and some initial studies of the mechanisms involved are presented in this Chapter. The data indicate significant upregulation of miR-223 and the

transcription factors c/EBPε and C/EBPβ in the induction of differentiation and indicate that this model has great potential for future detailed studies. Interestingly, data using a mIL-3 mutant developed in our group which is specific for signaling via IL-3Rα SP1 establishes an important role for signaling via the IL-3Rα SP2 isoform in the differentiation of this conditionally-immortalized GM progenitor.

Collectively, the data suggest that the mechanisms involved in the induction of differentiation via the IL-3-driven, steady state, CD44-induced and ATRA-induced pathways are separate but they likely invoke a common granulocytic differentiation pathway which involves upregulation of C/EBPε, C/EBPβ and miR-223.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Chemicals

All reagents were of analytical grade, or were of the highest grade obtainable.

2.1.2 Antibiotics

Penicillin	Sigma
Streptomycin	Sigma
Gentamicin sulphate	Sigma
Puromycin	Sigma
Gentamicin (G418)	Sigma

2.1.3 Antibodies

Rat anti-mouse IL-3Rα monoclonal antibody (5B11)	BD Pharmingen
Rat anti-mouse β_{IL-3} /βc monoclonal antibody (AIC2)	MBL
Monoclonal Anti-human IL-3 Rα Antibody (MAB301)	R&D Systems)
Purified Mouse Anti-Human CDw131 (hβc) (3D7)	BD Pharmingen
Rabbit anti-mouse IL-3Rα (sc-681)	Santa Cruz
Goat anti-rabbit IgG HRP (sc-2004)	Santa Cruz
Monoclonal anti-β actin (AC-74)	Sigma

Rat anti- mouse F4/80 antigen-FITC	Serotec
Rat Anti-mouse CD11b (Mac-1 α chain) -PE	BD Pharmingen
Rat anti-mouse Gr-1 mAb-FITC	Caltag Laboratories
Phycoerythrin (PE) anti-mouse FcεRIα (Mar-1)	eBioscience
Mouse Anti-Human CD11b (Mac-1)-PE	BD Pharmingen
Purified Mouse Anti-Human CD15 - FITC	BD Pharmingen
Phospho-Jak2 (Tyr1007/1008) (C80C3) Rabbit mAb	Cell signaling
Jak2 (D2E12) Rabbit mAb	Cell signaling
Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb	Cell signaling
Stat5 Antibody	Cell signaling
Phospho-Akt (Ser473) (D9E) Rabbit mAb	Cell signaling
Akt (pan) (C67E7) Rabbit mAb	Cell signaling
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb	Cell signaling
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell signaling
Goat anti-rabbit IgG HRP	Cell signaling

2.1.4. Kits

RNeasy Mini Kit	Qiagen
QIAshredder	Qiagen

Omniscript RT Kit	Qiagen
HotStarTaq Master Mix Kit	Qiagen
HotStar HiFidelity Polymerase Kit	Qiagen
miRNeasy Mini Kit	Qiagen
miScript Reverse Transcription Kit	Qiagen
miScript SYBR Green PCR Kit	Qiagen
QIAGEN Plasmid Midi and Maxi Purification Kits	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
NucleoSpin Extract II Kit (PCR clean-up Gel extraction)	Macherey-Nagel
pGEM-T Easy Vector Systems Kit	Promega
KAPA HiFi PCR Kit	KAPA Biosystems
Illustra Templiphi 100 Amplification kit	GE Healthcare
EZ-Vision one, DNA Dye as Loading Buffer, 6X	Amresco
Platinum Taq DNA Polymerase High Fidelity	Invitrogen
NuPAGE 4-12% Bis-Tris 1.0 mm 10 well gel	Invitrogen
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific

2.1.5. Oligonucleotides

Table 2.1: Primers used for RT-PCR and quantitative real time PCR

Gene	Primer sequence	
mIL-3Rα SP1 or SP2	F	5'-ATGGCCGCCAACCTGTGGCTC-3'
	R	5'-GCAGGACCGAGCCGCACATCA-3'
hIL-3Rα SP1 and SP2	F	5'-CTGTCTCCTGCAAACGAAGG-3'
	R	5'-GTTGACGCCTGTTGGCAACG-3'
Mouse β-actin	F	5'-GTGGGCCGCTCTAGGCACCA-3'
	R	5'-CGGTTGGCCTTAGGGTTCAGGGGGG-3'
Human β-actin	F	5'-CCTCGCCTTTGCCGATCC-3'
	R	5'-GGATCTTCATGAGGTAGTCAGTC-3'
Mouse GATA-1	F	5'-CATTGGCCCCTTGTGAGGCCAG-3'
	R	5'-CGCTCCAGCCAGATTCGACCC-3'
Mouse GATA-2	F	5'-CGACACACCACCCGATACCCACCTAT-3'
	R	5'-CGCCTACGCCATGGCAGTCACCATGCT-3'
Mouse PU.1	F	5'-GAGTTTGAGAACTTCCCTGAG-3'
	R	5'-TGGTAGGTCATCTTCTTGCGG-3'
Mouse c-Myb	F	5'-GAGCTTGTCCAGAAATATGGTCCGAAG-3'
	R	5'-GGCTGCCGCAGCCGGCTGAGGGAC-3'

Mouse AML1	F	5'-CCAGCAAGCTGAGGAGCGGCG-3'
	R	5'-CCGACAAACCTGAGGTCGTTG-3'
Mouse Ikaros	F	5'-CACTACCTCTGGAGCACAGC-3'
	R	5'-TCTGAGGCATAGAGCTCTTA-3'
Mouse Id1	F	5'-TCAGGATCATGAAGGTCGCCAGTG-3'
	R	5'-TGAAGGGCTGGAGTCCATCTGGT-3'
Mouse C/EBPα	F	5'-TGCGTCTAAGATGAGGGAGT-3'
	R	5'-GATTAGGAGCCCTCCACCTC-3'
Mouse C/EBPβ	F	5'-CGCCCGCGCACCACGACTTCCTCT-3'
	R	5'-CGTCGCTCAGCTTGTCCACCGTCTT-3'
Mouse C/EBPδ	F	5'-CGGGCAGAGGGCGGGTCGTTCC-3'
	R	5'-GCCGGCCGCTTTGTGGTTGCTGTT-3'
Mouse C/EBPε	F	5'-TGTGGCGGTGAAAGAGGAGCCT-3'
	R	5'-GGCTCAGCTGCAGCCCCC-3'
Mouse NFYA	F	5'-TGCAGCCTCTGATTGGGTTTCG-3'
	R	5'-TCCTCCTGAGTTGACCACATTCC-3'
Mouse UBC	F	5'-AAGAGAATCCACAAGGAATTGAATG-3'
	R	5'-CAACAGGACCTGCTGAACACTG-3'
Mouse HPRT	F	5'-CACAGGACTAGAACACCTGC -3'

	R	5'-GCTGGTGAAAAGGACCTCT -3'
Mouse NFI-A	F	5'-AACCGGCCCCAATTTTTCTCT 3'
	R	5'-AGGCTGCTGGATGATGGGTGAC- 3'
Mouse Gfi-1	F	5'-CCTTGGCCTGGGAACCTACC-3'
	R	5'-GGGGGCCTCCAAAAGTCCTC-3'
Mouse G-CSFR	F	5'-GCTCCAGCGCTGCATCTAAAG-3'
	R	5'-TGGGCTGGGTCTGGCACATC-3'
Mouse M-CSFR	F	5'-AATGGCAGTGTGGAATGGGATGG-3'
	R	5'-GTGGGGGCTCTGGGTGGACTC-3'
Human β common	F	5'-GCACCGGCTACAACGGGATCT-3'
	R	5'-TCCCCGAATCCTACAGGGAAC-3'
Mouse CBAP	F	5'-TTCGCTCTACTCGGTCTGGTTCAC-3'
	R	5'-GGGACTCTCTTTGCTATGGGTTTG-3'
Human CBAP	F	5'-CCAAGGACTTTGTCTTCTCTTTGC-3'
	R	5'-ACTTTCTTCGCTCTCCGTTCG-3'
Mouse OPN	F	5'-TGATGAACAGTATCCTGATGCCAC-3'
	R	5'-GCTTTGGAACTTGCTTGACTATCG-3'
Human OPN	F	5'-TGCAGCCTTCTCAGCCAAAC-3'
	R	5'-TTACAGGGAGTTTCCATGAAGCC-3'

Mouse CD44	F	5'-CCAGAAGGCTACATTTTGCACAC-3'
	R	5'-TGGAGTCCTTGGATGAGTCTCG-3'
Human CD44	F	5'-GGGTTCATAGAAGGGCATGTGG-3'
	R	5'-TCTCCTTTCTGGACATAGCGGG-3'
Mouse βc	F	5'-TATGTGCTAAAAGGTAGTGTTTGC-3'
	R	5'-TGTGCTCAATGAATGAGTAAGCC-3'
Mouse β_{IL-3}	F	5'-ATGTGTGCTAAAAGATTACCTCTAT-3'
	R	5'-AAAGTGTGATCAATGTATTTGGGT-3'
Mouse GM-CSF Rα	F	5'-AGGAAGCCCCCTGTCTCAG-3'
	R	5'-CCTTCCTGTCAGTCACGTTG-3'

2.2 Methods

2.2.1 Cell culture

Unless otherwise stated, the cytokines used for cell growth were produced in insect cells (see below) and not further purified. Previous work in our laboratory verified that the unpurified preparations of the cytokines used had the same biological activities as purified preparations. The SCF used was conditioned medium from the CHO (SCF) cell line (see below).

2.2.1.1 Expression of cytokines

The *flash*BAC system (Oxford Expression Technologies Limited) was used for the production of recombinant baculoviruses. Baculoviruses are insect viruses, predominantly infecting insect larvae of the order Lepidoptera (butterflies and moths) (van Regenmortel, 2000). A baculovirus expression vector is a recombinant baculovirus that has been genetically modified to contain a foreign gene of interest, which can then be expressed in insect cells under control of a baculovirus gene promoter. The most commonly used baculovirus for foreign gene expression is AcMNPV (Smith and Crook, 1993). Recombinant baculoviruses were produced by cotransfection of *Sf9* cells with *flash*BAC Gold^{TM} plus the transfer vector pBacPAK8 containing the gene encoding the protein to be expressed. Briefly, the pBacPAK8 plasmid DNA was diluted in $T_{10}E_1$ buffer to 100ng/µl and 2 µl of this DNA was added to 400 µl of serum free medium, 2 µl of *flash*BAC Gold DNA and 2 µl of transfection reagent. The solution was kept at RT for 30 minutes after gentle mixing. Two 4.5cm^2 wells in a 6 well plate were seeded with $0.75x10^5$ *Sf9* cells growing in TNM-FH with 10% FBS. After the cells adhered to the surface, they were washed twice in serum-free TNM-FH. The DNA mixture was

added gently and immediately to the cells. After incubating the plates for 5 hours, 1 ml of fresh TNM-FH with 10 % FBS was added to each well to give a final concentration of 5% FBS. Incubation of the plates was then continued at 27°C for 5 days, and the medium containing the virus collected and any cells removed by centrifugation at 1000 rpm for 20 minutes. The virus titre was then amplified by several more passages using *Sf9* cells in serum containing medium.

The virus stock was then used to infect Hi5 cells (Invitrogen) for the production of expressed protein. Cells ($5x10^7$) were seeded into 175-cm^2 flasks in Ex-Cell™ 405. 10 ml of virus in serum-containing TNM-FH medium was added and the cells incubated with virus for 1 hour, after which time the virus was drained off and the cells washed three times to remove traces of serum. 35 ml of Ex-Cell™ 405 medium was added and incubation continued for a further 72 hours. The supernatant containing the secreted recombinant protein was centrifuged at 1000 rpm for 20 minutes and stored frozen in aliquots.

SCF-conditioned media was produced by culturing the CHO (SCF) cell line (kindly provided by Prof. M.P. Kamps) in OptiMEM medium supplemented with 10% v/v FBS, penicillin-streptomycin-glutamine solution and 2-mercaptoethanol. The cells are adherent and grow quickly. The media has to be changed every day. SCF-conditioned media stock was harvested and stored frozen in aliquots at -80 °C.

2.2.1.2 Cell lines and cell culture

Cells were grown in the following media: CTLL-2, RPMI 1640, 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1% v/v mIL-2; B4.1 (*Hox*-2.4 mouse myelomonocytic cells), DMEM, 10% FCS, 2% v/v mIL-3; FDCP-mix, Iscove's MEM, 20% horse serum, 2% v/v mIL-3, 0.3% w/v $NaHCO_3$, osmolarity adjusted to 0.32

osmol/L; FDCP-1, RPMI 1640, 10% FCS, 0.1% v/v mIL-3, 2 mM L-glutamine, 10 mM NaCl, 1 mM sodium pyruvate, 0.124 mM monothioglycerol; GB2, high glucose DMEM, 10% FCS, 0.05 mM 2-mercaptoethanol, 0.1 mM L-asparagine; WEHI-3B D^+/D^-, M1 and HL60, DMEM, 10% FCS; TF1, RPMI 1640, 10% FCS, 4 mM L-glutamine, 0.1% v/v hIL-3; NB4, RPMI 1640, 10% FCS, 2 mM L-glutamine; GM-CSF ER-HoxB8 progenitors, RPMI1640, 10% FBS, 1% Pen-Strep-Glut (PSG, Gibco) 1% v/v GM-CSF, 1 μM β-estradiol (Sigma); SCF ER-HoxB8 progenitors, OptiMem I medium containing 10% FBS, 1% PSG, 1% v/v SCF (stem cell factor), 30uM beta mercaptoethanol, and 1 μM β-estradiol (Sigma).

Cells were cultured in 5% CO_2, except for GB2, WEHI-3B D+/D- and M1 cells which were cultured in 10% CO_2. The growth medium for all cell lines was supplemented with 60 μg/ml benzylpenicillin, 100μg/ml streptomycin and 10 μg/ml gentamycin.

2.2.1.3 Stable transfection

Stable transfections were used to derive cell lines expressing relevant receptors. cDNAs encoding mouse and human IL-3Rα SP1 and SP2 were cloned into the pEFIRES-N (G418) expression vector and cDNAs for mβc, $m\beta_{IL\text{-}3}$ or hβc cloned into pEFIRES-P (puromycin). Restriction digestion was used to linearise 30 μg of a pEFIRES DNA construct, and DNA was purified by phenol/chloroform extraction, precipitated with ethanol and washed twice with 70% ethanol before air-drying. The DNA pellet was dissolved in 50 μl plain medium and mixed with approximately 10^7 cells resuspended in 320 μl growth medium before electroporation. Transfection of expression constructs into cells was done with a Bio-Rad Gene Pulser Xcell Electroporation System with settings of 290 V and 950μF capacitance for M1, NB4 cells and 260 V and 1050 μF for FDCP-mix cells. Electroporated cells were allowed to stand for 10 minutes before being

transferred to 30 ml growth medium. 2 days after transfection, selection was carried out using 0.4 mg/ml G418 (M1, FDCP-mix), 0.25 mg/ml G418 (NB4) and/or puromycin at 0.006 mg/ml (M1), 0.0005mg/ml (NB4) and 0.001 mg/ml (FDCP-mix). As many as possible stable transfectants were recovered yielding polyclonal populations of cells.

2.2.1.4 Long term storage and thawing of cells

Cell lines were stored in liquid nitrogen according to standard techniques. Cells growing in logarithmic phase were centrifuged (1500 rpm for 5 minutes at 4 °C) and resuspended at (>lxl0^6) in cold freezing medium (70% medium, 10% v/v dimethyl sulfoxide (DMSO), 20% FBS). 1 ml aliquots were dispensed into sterile pre-chilled cryotubes. The cryotubes were placed into a NALGENE Cryo Freezing Container (Nalge Nunc), an isopropanol rate cooling device, and placed at -80 °C for at least 24 hours before the vials were transferred to liquid nitrogen storage tanks. For recovery, stored cells were thawed rapidly in a 37 °C waterbath and transferred to a 15 ml sterile polypropylene tube. The cell suspension was mixed with 9 ml of growth media added dropwise over 5 minutes whilst mixing. Cells were centrifuged at 1500 rpm for 5 minutes at room temperature and resuspended in 10 ml fresh growth medium.

2.2.2 RNA Isolation and RT- PCR

2.2.2.1 RNA Isolation

Total RNA was prepared using a Qiagen RNeasy mini kit. Briefly, up to 1 x 10^7 cells, depending on the cell line, were disrupted using Buffer RLT. One volume of 70% ethanol was then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample was then applied to the RNeasy Mini spin column and the RNA bound to the membrane. The column was washed with Buffer

RPE to remove contaminants and the high-quality RNA eluted in RNase-free water (Qiagen). All binding, washing, and elution steps were performed by centrifugation in a microcentrifuge.

2.2.2.2 cDNA synthesis

cDNA was synthesized using a Qiagen Omniscript RT kit with anchored Oligo(dT)$_{20}$ primers. Anchored Oligo (dT)$_{20}$ Primer (Invitrogen) is a mixture of 12 primers, each consisting of a string of 20 deoxythymidylic acid (dT) residues followed by two additional nucleotides represented by VN, where V is dA, dC, or dG and N is dA, dC, dG or dT. The VN "anchor" allows the primer to anneal only at the 5′ end of the poly (A) tail of mRNA, providing more efficient cDNA synthesis.

Briefly, a fresh master mix was prepared (Table 2.2) and kept on ice. The primers used are listed in Table 2.1. The reaction mixture was carefully vortexed for no more than 5 seconds, then centrifuged briefly and stored on ice. The template RNA was added and mixed carefully by vortexing for no more than 5 seconds. The reaction mixture was centrifuged briefly and incubated for 60 minutes at 37°C.

Table 2.2: Reverse transcription reaction components

Component	Volume/reaction	Final concentration
Master mix		
10xBuffer RT	2µl	1x
dNTPMix (5mM each dNTP)	2µl	0.5mM each dNTP
Oligo-dT primer (10µM)	2µl	1µM
RNase inhibitor (10 units/µl)	1µl	10 units (per 20µl reaction)
Omniscript Reverse Transcriptase	1µl	4 units (per 20µl reaction)
RNase-free water	Variable	
Template RNA		
Template RNA	Variable	Up to 2 µg* (per 20µl reaction)

* This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA analyzed.

2.2.2.3 Polymerase Chain Reaction (PCR)

The prerequisite for a PCR to be used for quantification is that PCR product is measured within the exponential phase of the PCR reaction, where the amount of amplified target is directly proportional to the input amount of target. Therefore, quantification of gene expression by semi-quantitative RT-PCR was carried out during the exponential (log) phase of the PCR reaction and the plateau phase was avoided. The exponential phase of the PCR reaction was determined empirically by amplifying equivalent amounts of cDNA over different number of PCR cycles. The kinetic analysis was performed by amplifying PCR products over different number of PCR cycles and showed that PCR reaction was exponential between cycles 25 and 30.

PCR was performed using a Qiagen HotStarTaq Master mix kit. Briefly, the HotStarTaq Master Mix was mixed by vortexing briefly and then dispensed into PCR tubes. The appropriate volume of the diluted primer mix was distributed into the PCR tubes containing the Master Mix. The primers used are listed in the Table 2.1. Template DNA (<1 µg/50 µl reaction) was added to the individual PCR tubes. The conditions used for PCR were 30 cycles of 94 °C for 30 seconds, 50-68 °C (approximately 5 °C below the T_m of the primers) for 45 seconds and 72 °C for 1 minute using a PTC-200 Peltier thermal cycler (MJ Research, Watertown MA).

2.2.3 MiRNA preparation and real-time PCR

2.2.3.1 MiRNA preparation

miRNA was prepared using a miRNeasy Mini kit (Qiagen) (Fig.2.1). Briefly, cell samples were disrupted by adding QIAzol Lysis reagent and were homogenized using a QIAshredder homogenizer. After the addition of chloroform, the homogenate was

separated into aqueous and organic phases by centrifugation. The RNA partitioned to the upper, aqueous phase, while the DNA partitioned to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was collected, and ethanol added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The sample was then applied to the RNeasy Mini spin column. The RNA bound to the membrane and phenol and other contaminants were efficiently washed away. High-quality RNA was then eluted in RNase-free water (Qiagen).

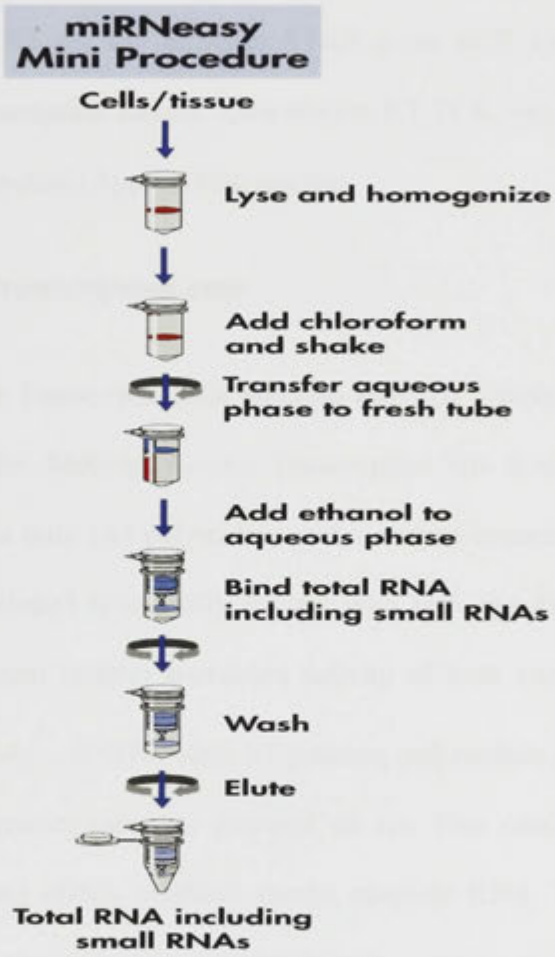


Figure 2.1: miRNeasy mini procedure.

2.2.3.2 Quantitative real-time RT-PCR

The detection and quantification of miRNAs was carried out by quantitative real time RT-PCR using the miScript reverse transcription kit, miSript primer assays and miScript SYBR green PCR kit (Qiagen) according to the manufacturer's instructions. Qiagen's Omniscript RT kit and miScript SYBR green PCR kit were employed in quantification of transcription factors. Quantitative RT-PCR was conducted with the 7500 Real time PCR system (Applied Biosystems).

2.2.3.2.1 Reverse-transcription step

The miScript Reverse Transcription Kit includes miScript Reverse Transcriptase Mix and miScript RT Buffer. MiScript Reverse Transcriptase Mix is an optimized blend of enzymes comprising a poly (A) polymerase and a reverse transcriptase. MiScript RT Buffer has been developed specifically for use with miScript Reverse Transcriptase Mix. This buffer system enables maximum activity of both enzymes. MiScript RT Buffer also contains Mg^{2+}, dNTPs, oligo-dT primers, and random primers. Briefly, the reverse-transcription master mix was prepared on ice. This contains all components required for first-strand cDNA synthesis except template RNA. Template RNA was added to each tube containing reverse-transcription master mix and the reactions incubated for 60 minutes at 37°C. The reactions were heated for 5 minutes at 95°C to inactivate miScript Reverse Transcriptase Mix, placed on ice and then used for real-time PCR (Fig.2.2).

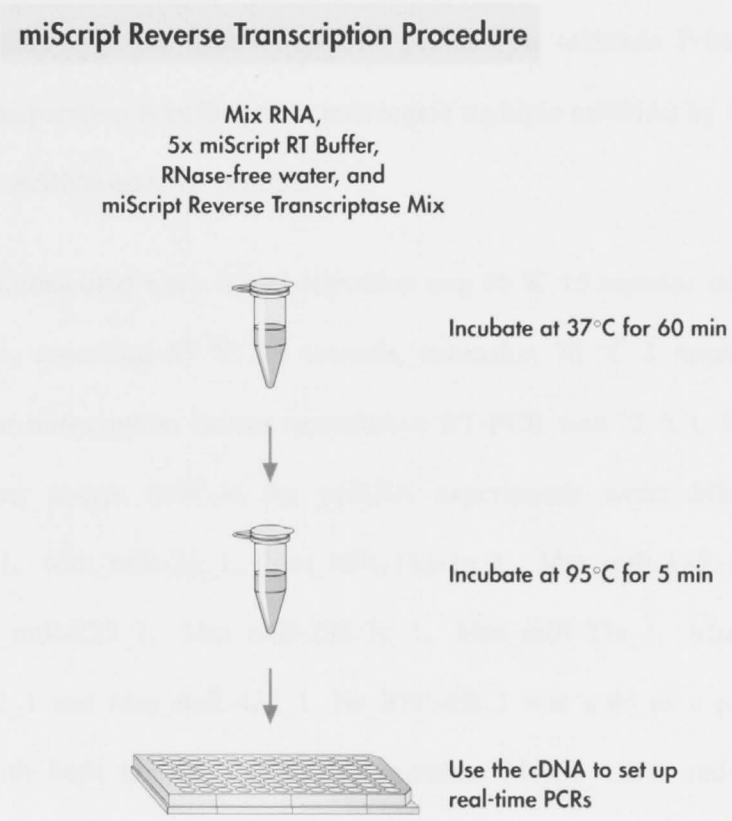


Figure 2.2: MiScript Reverse Transcription procedure.

2.2.3.2.2 Real-time PCR step

The cDNA serves as the template for real-time PCR analysis using the miScript Primer Assay in combination with the miScript SYBR Green PCR Kit (Fig.2.3). MiRNAs are amplified using the miScript Universal Primer, which primes from the universal tag sequence, together with the miRNA-specific primer (the miScript Primer Assay). A single cDNA preparation is sufficient to interrogate multiple miRNAs by real-time PCR using different miRNA-specific primers.

The PCR conditions used were: initial activation step 95 °C 15 minutes, denaturation 94 °C 15 seconds, annealing 55 °C 30 seconds, extension 70 °C 1 minute (extension temperature for transcription factors quantitative RT-PCR was 72 °C), 40 cycles. The miScript primer assays used in the miRNA experiments were: Mm_miR-15a_1, Mm_miR-16_1, Mm_miR-21_1, Mm_miR-142-3p_1, Mm_miR-155_1, Mm_miR-181a_1, Mm_miR-223_1, Mm_miR-292-3p_1, Mm_miR-23a_1, Mm_miR-27a_1, Mm_miR-24-2_1 and Mm_miR-424_1. Hs_RNU6B_1 was used as a reference gene compatible with both human and mouse samples. Mouse UBC and HPRT were employed as reference genes for the quantitative real time RT-PCR of transcription factors. Details of the primers used for reference genes and transcription factors are listed in Table 2.1. The data were analyzed using the comparative C_T method ($\Delta\Delta C_T$ method). To calculate the changes for SCF ER-hoxb8 cells undergoing differentiation, the fold-difference of miRNA or transcription factor expression was calculated for cells grown without estrogen (differentiating) relative to cells grown with estrogen (non-differentiating).

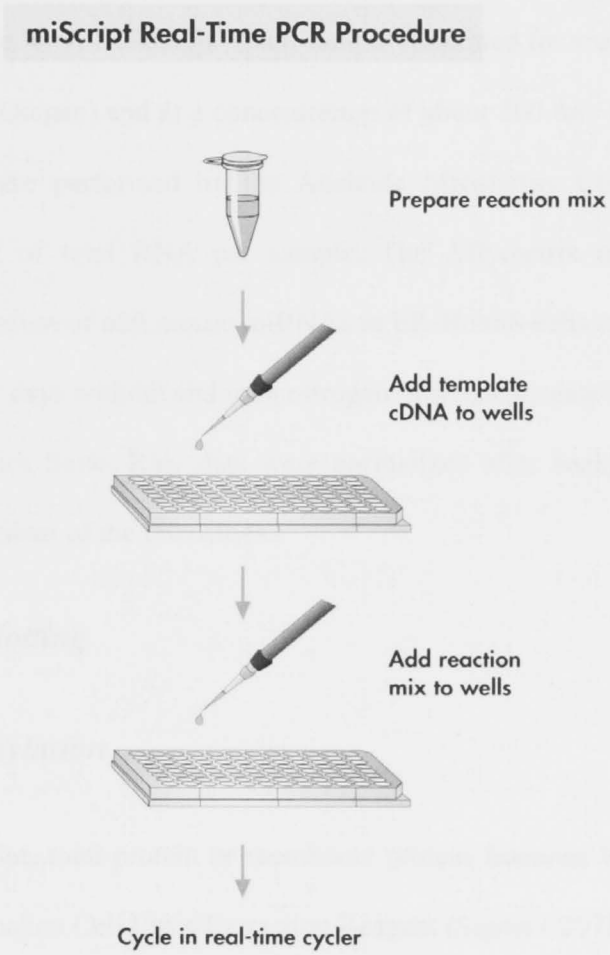


Figure 2.3: MiScript real-time PCR procedure.

2.2.4 MiRNA microarrays

MiRNA was prepared using a miRNeasy Mini kit (Qiagen). RNA quality was assessed by the Agilent 2100 Bioanalyzer prior to running microarrays. The sample profiles were analyzed by the software to generate an RNA Integrity Score (RIN). Up to 12 samples were run on a single RNA Nanochip. Each sample submitted for analysis was in 3 µl of RNase-free water (Qiagen) and at a concentration of about 200 ng / µl. The Affymetrix miRNA Arrays were performed by the Adelaide Microarray Centre. These arrays required only 1µg of total RNA per sample. The Affymetrix miRNA microarray analyzed the expression of 609 mouse miRNAs in ER-Hoxb8 cells cultured with mIL-3 or mGM-CSF for 2 days without and with estrogen. Microarray data was analyzed using the Partek Genomics Suite. Raw data were normalized after background subtraction using the global median of the intensities.

2.2.5 Western blotting

2.2.5.1 Deglycosylation

For the Western blot, total protein or membrane protein fractions were prepared with CelLytic-M Mammalian Cell Lysis/Extraction Reagent (Sigma C2978) or CelLytic MEM protein extraction kit (Sigma CE0050) according to the manufacturer's instructions. Briefly, for deglycosylation, 80 µg of membrane protein fraction in 19.5 µl 1x incubation buffer (20 mM sodium phosphate, 0.02% NaN_3), was treated with SDS (0.1% final) and β-mercaptoethanol (50 mM final), and the proteins denatured by heating at 100 °C for 5 minutes. When cool, NP-40 (0.75% final) and 3U of N-Glycosidase F (PNGase F, Roche) were added and the mixture incubated for 3 hours at 37 °C.

2.2.5.2 Western blot analysis

Western blotting was performed using an Invitrogen 4-12% NuPAGE Novex pre-cast gel system according to the manufacturer's instructions. Rabbit anti-mouse IL-3Rα (sc-681) antibodies and goat anti-rabbit IgG HRP (sc-2004) were used (both from Santa Cruz) at a 1:1000 dilution for primary antibody and a 1:20,000 dilution for secondary antibody. Monoclonal anti-β actin (AC-74, Sigma) was used at a 1:5000 dilution. The membranes were washed 5 times in TBS containing 0.1% tween-20. Protein bands were detected using ECL Plus Chemiluminescent substrate according to the manufacturer's instructions.

2.2.5.3 BCA protein assays

The BCA Working Reagent was prepared by mixing 50 parts of Bicinchoninic Acid Solution with 1 part of Copper (II) Sulfate Solution. Mixing was continued until the BCA Working Reagent was light green in color. BSA protein standards ranging from 0.2mg/ml to 8.0mg/ml were prepared and 2 ml of the BCA Working Reagent were added to 0.1 ml of each BSA protein standard, blank, and unknown sample. The samples were vortexed gently for thorough mixing and incubated at 37 ° C for 30 minutes. The absorbance of the solution at 562 nm was measured by NanoDrop ND-1000 spectrophotometer. Protein concentrations were determined from the standard curve for the BSA protein standards.

2.2.5.4 Gel staining

GelCode Blue Stain Reagent stains only protein and allows bands to be viewed directly on the gel for the use of total protein stains as loading control. After electrophoresis, gels were washed in ultrapure water with gentle shaking for 15 minutes. GelCode Blue

Stain Reagent was added and the gel was observed for approximately 1 hour to monitor protein band development. To destain the gel, the GelCode Stain Reagent was replaced with ultrapure water which was changed several times over 1-2 hours. Protein bands were visualized using the Fuji luminescent image analyser (Las-1000 plus, Fujifilm).

2.2.5.5 Analysis of signal transduction pathways

The phosphorylated and total Jak2, STAT5, Akt and Erk1/2 were investigated in SCF ER-Hoxb8 and BaF3 cells. All the antibodies used were from Cell Signaling (mouse anti-phosphorylated $Jak2^{Tyr1007/1008}$, $STAT5^{Tyr694}$, Akt^{Ser473}, $Erk1/2^{Thr202/Tyr204}$ and anti-Jak2, STAT5, Akt, Erk1/2). SCF ER-Hoxb8 and BaF3 cells were treated with 1% mIL-3 or mutant mIL-3 at the indicated times. The cells were harvested and washed once with cold PBS with 1mM Na_3VO_4. Then the cell lysates were prepared with the cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstain A, 10 mM NaF, 1 mM PMSF, 4mM AEBSF, 250μM Cathepsin G inhibitor I) at 4 °C 0.5 hour and the lysates were cleared by centrifugation for 10 minutes at 4 °C and 100,000xg. The cleared lysates were subjected to SDS-PAGE using Invitrogen's Norvex Nupage reducing gel system. The Western blot for phosphorylated and total Jak2, STAT5, Akt, Erk1/2 was conducted according to the protocol provided by Cell Signaling. After probing with the phosphorylated-antibodies, the same blot was stripped and re-probed for detection of total proteins.

2.2.5.6 Antibody stripping

The Millipore Re-Blot Plus Western Blot Antibody Stripping Solution is effective for removal of antibodies from Western blots that have been developed with

chemiluminescence and was used in the work described in this thesis. Mild or Strong Antibody Stripping Solution 10x was diluted 10-fold with distilled water to obtain a 1x solution. Blots were submerged in stripping solution in a plastic tray and incubated with gentle mixing for 15 minutes at room temperature. Blots were washed twice for 5 minutes with blocking buffer before reprobing with antibodies.

2.2.6 Flow cytometry

Approx 10^6 cells were harvested and washed with ice-cold FACS buffer (2% FCS, 0.02% NaN_3 in PBS). For surface staining, cells were stained for 30 min in the dark at 4°C. The following antibodies were used: Serotec, anti-mouse F4/80-FITC; BD Pharmingen, anti-mouse Mac-1-PE; Caltag Laboratories, anti-mouse Gr-1-FITC; eBioscience, anti-mouse Mar-1-PE (FcεRIα); BD Pharmingen, anti-Human CD11b (Mac-1)-PE; BD Pharmingen, anti-Human CD15-FITC; 7-AAD. Data were acquired on a FACSort cytometer and analyzed using FlowJo software. Two steps of flow cytometry were used to detect the ectopically expressed human IL-3Rα SP1 or SP2 with human βc in NB4 cells, as well as mouse IL-3Rα SP1 or SP2 with mouse $\beta_{IL\text{-}3}$ or mβc in M1 cells. Cells were harvested and washed with cold PBS, and then cells were incubated with 1:200 dilution of primary antibody in FACS incubation buffer (0.2% BSA, 5% FBS in cold PBS) for 1 hour. After 3 washes with FACS washing buffer (0.2% BSA in cold PBS), the cells were then incubated with secondary antibody (1: 500 dilution of the relevant isotype of IgG or IgM in FITC or PE, 1:2000 dilution of SA-PE) for 0.5hour. Cells were washed three times with FACS washing buffer before being subjected to FACS. The same concentration of the relevant isotypes of immunoglobin of primary antibodies was used as the negative control. The primary monoclonal antibodies used were: MAB301 (for detection of hIL-3Rα SP1 and SP2, R&D Systems), 3D7 (CDw131, for detection of hβc, BD Pharmingen), 5B11 (for detection of mIL-3Rα, BD

Pharmingen), and AIC2 (for detection of mouse β_{IL-3}, or mβc, MBL Medical and Biological Laboratories).

2.2.7 Morphological analysis

M1 or NB4 cells were cultured using a Lab-Tek II chamber slider system (Nalge Nunc) in growth medium for 4 days with and without mIL-3 or hIL-3. SCF ER-Hoxb8 cell was cultured with mIL-3, mGM-CSF or SCF using a Lab-Tek II chamber slider system for 4 days with or without estrogen. May-Grunwald-Giemsa staining was used. This staining method is based on the electrostatic interaction between dye and target molecules. The staining solutions contain methylene blue (a basic dye), related azures (also basic dyes) and eosin (an acidic dye). Briefly, the fixed slide was stained for 5 minutes in May-Grunwald stain diluted with an equal volume of distilled water. The slide was transferred without washing into Giemsa stain diluted with 9 volumes of distilled water for 30 minutes, then washed in distilled water and allowed to dry. After May-Grunwald-Giemsa staining, cell morphology was examined under a 100x oil objective using light microscopy.

2.2.8 Proliferation assays

Growth responses of SCF ER-Hoxb8 and Ba/F3 cells to mIL-3 or mIL-3 E23A were measured in triplicate by [^{3}H] incorporation. The proliferation was measured after 2 days incubation. Briefly, a two-fold serial dilution of a cytokine (mIL-3 or mIL-3 E23A) was performed in triplicate in round-bottomed 96-well plates. The cells were washed four times with factor-free growth media and 50µl of $4x10^5$ cells /ml were added into each well of the 96-well plate. The cells were incubated in a 95% CO_2 incubator at 37 °C for 44 hours. 0.5µCi of tritiated thymidine was added to each well and the incubation continued for a further 4 hours. The cells were then harvested on to

glass fibre filters and the filters were washed three times with distilled water, dried, covered with 100µl of scintillation fluid and counted using a Packard Tri-Carb 1900CA liquid scintillation counter.

2.2.9 RNA interference

RNA interference (RNAi) is a highly evolutionally conserved process of post-transcriptional gene silencing (PTGS) by which double stranded RNA (dsRNA), when introduced into a cell, causes sequence-specific degradation of homologous mRNA sequences. On entering the cell, long dsRNAs act as a trigger of the RNAi process. They are first processed by the RNAse III enzyme Dicer in an ATP-dependent reaction. Dicer processes dsRNAs into 21-23 nt short interfering RNA (siRNA) with 2-nt 3' overhangs. siRNA can also be synthesized outside the cell and then introduced into cells. The siRNAs are incorporated into the RNA-inducing silencing complex (RISC) which consists of an Argonaute (Ago) protein as one of its main components. Ago cleaves and discards the passenger (sense) strand of the siRNA duplex leading to activation of the RISC. The remaining guide (antisense) strand of the siRNA guides RISC to its homologous mRNA, resulting in the endonucleolytic cleavage of the target mRNA (Fig.2.4).

The procedure used for RNA interference in the present work is described below. Three million log phase NB4 cells were washed twice and resuspended in 500 µl of electroporation buffer containing 0.5 nmol (final concentration 1µM) of hCD44 siRNAs (Ambion), hβc siRNAs (Ambion) or negative siRNA control in a 4-mm gap cuvette. The mixture was then electroporated with settings of 260 volts and 1050 µF using a Gene Pulser Xcell electroporation apparatus (Bio-Rad). Twenty-four hours after

transfection, cells were treated with 1 μM ATRA. The cells were harvested for real-time RT-PCR to check the mRNA expression of target genes.

Using the electroporation condition described above, a transfection efficiency of 80% or higher was consistently achieved without significant reduction of viability. After NB4 cells were treated with ATRA for 4 days, the expression of surface markers were measured by FACS.

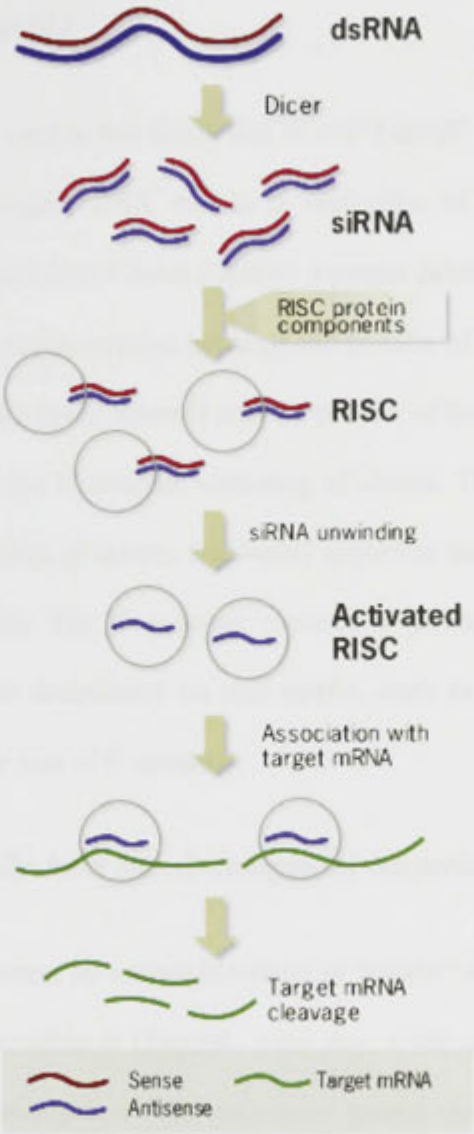


Figure 2.4: The mechanism of RNA interference.

2.2.10 Recombinant DNA techniques

2.2.10.1 Preparation of bacterial cells

2.2.10.1.1 E. coli Top10F'

The main bacterial cell used in this thesis was *E. coli* Top10F' (Grant *et al.*, 1990). This strain was from Invitrogen, USA and is a derivative of Hanahan's strain, DH5 (Hanahan, 1985). The *lacZ*ΔM15 locus contains a partial deletion of the β-galactosidase gene that enables α-complementation between the portion of the β-galactosidase gene carried on a pUC or M13-based plasmid and the portion of the chromosome of the host bacterial cell, allowing the blue-white screening of clones. The presence of the *recA1* allele increases the stability of inserts, and *endA1* improves the quality of plasmid DNA purified from this strain. The F' episome contains a tetracycline resistance marker, allowing Top10F' to be maintained on rich media, such as LB or SOB, containing tetracycline, without the loss of F' episome.

2.2.10.1.2 Chemically-heat shock-competent bacterial cells

To select for the F' episome, an overnight culture of Top10F' was grown in a small flask in the presence of tetracycline at 15μg/ml. Next day, 1/100 dilution of the culture was made in LB without tetracycline. The cells were grown until the culture reached an optical density (OD) between A_{600} 0.4 – 0.6. The cells were collected by centrifugation at 3700 rpm for 8 minutes at room temperature and resuspended gently in half the original volume, of ice-cold 50mM $CaCl_2$. The cells were centrifuged again and resuspended in 1/10 original volume, of 50mM $CaCl_2$. Sterile glycerol was added to a final concentration of 15% v/v. Chemically-competent bacterial cells were frozen in liquid nitrogen in 100 or 200 μl aliquots and stored at -70°C until required.

2.2.10.1.3 Transformation of chemically heat-shock competent cells

A 50 µl aliquot of Top10F' chemically-competent bacterial cells was thawed on ice and incubated for 30 minutes with 5-10 µl of plasmid DNA. The bacterial sample was heat shocked for 45 seconds at 42°C, cooled on ice for 2 minutes and incubated for 45-60 minutes at 37°C with preheated 200 µl LB media with gentle shaking at 160 rpm. Then the bacteria were spread onto LB-agar plates containing an appropriate antibiotic for selection of the plasmid and incubated overnight at 37°C.

2.2.10.2 Mini preparation of plasmid DNA

DNA was prepared using the QIAprep DNA Miniprep kit (Qiagen), according to the manufacturer's instructions. Briefly, a single colony was inoculated into LB medium supplemented with the appropriate antibiotic (usually ampicillin at 50-100 µg/ml) and the culture grown overnight at 37 °C with shaking at 250rpm. The cells were harvested by centrifugation, and lysed by alkaline lysis. The lysate was neutralised and the cellular debris removed by centrifugation. The supernatant was passed through an anion-exchange resin containing positively-charged DEAE groups that bind the negatively-charged DNA molecules. The DNA was subsequently eluted using $T_{10}E_{0.1}$ buffer (10mM Tris-HCl, 0.1 mM EDTA, pH8.0).

2.2.10.3 Glycerol stock preparation

A single colony of bacterial cells was used to inoculate 10 ml of LB medium supplemented with 50-100µg/ml ampicillin and grown for 16 hours at 37°C with shaking at 250 rpm. Glycerol stocks were prepared by adding 850 µl of overnight culture to 150 µl of sterile glycerol, and were stored at -70°C.

2.2.10.4 Large scale preparation of plasmid DNA

Large amounts of DNA were prepared using the Qiagen midiprep or maxiprep kits according to the manufacturer's instructions. Briefly, bacterial cells from glycerol stocks were streaked on LB-agar plates at 37°C for single colonies. A single colony was inoculated into 50 ml or 200 ml of LB medium supplemented with 50-100 μg/ml ampicillin for DNA midipreps or maxipreps, respectively, and grown for 16 hours at 37°C with shaking at 270 rpm. Cells were harvested by centrifugation at 5000 rpm and lysed by alkaline lysis. After removal of the cell debris by centrifugation or filtration, the crude DNA was applied to an anion-exchange resin under low salt conditions. RNA, proteins and low molecular weight components were removed by a medium salt wash. The plasmid DNA was eluted by a high salt buffer, concentrated and desalted by isopropanol precipitation. The DNA precipitate was pelleted by centrifugation at 15000 x g at 4°C and washed with 70% ethanol. The pellet was dried at room temperature and resuspended in $T_{10}E_{0.1}$ buffer or milliQ water.

2.2.10.5 Quantitation of nucleic acids

The concentration of DNA was quantitated using a Nanodrop Spectrophotometer. The ratio between the OD readings at 260 nm and 280 nm (OD260/OD280) was used to estimate the purity of the DNA preparation. Pure preparations have OD260/OD280 ratios of 1.8, with lower readings indicating protein or phenol contamination.

2.2.10.6 Restriction enzyme digestion of DNA

Restriction endonucleases were supplied by New England Biolabs, Promega or MBI Fermentas. DNA digestion was performed according to the manufacturer's protocols and in the buffer specified for each enzyme. The DNA concentration in the final

reaction was kept at 50 to 100 ng/µl and glycerol was kept to below 5% (w/v) to minimise non-specific endonuclease activity. Bovine serum albumin (BSA) was routinely included in the reaction at 0.1 mg/ml.

2.2.10.7 Agarose gel electrophoresis

Electrophoresis was performed using agarose gels composed of 0.8- 2% (w/v) agarose dissolved in 1xTAE (Tris-Acetate electrophoresis buffer containing 40 mM Tris-acetate, 1 mM EDTA pH 8.0). The gels were submerged in a tank (Bio-Rad, USA) containing 1xTAE buffer and were electrophoresed at up to 100 V. AMRESCO's EZ-VISION fluorescent DNA reagent was used for band visualization. EnVISION DNA dye 6X Loading Buffer was diluted with 5 parts DNA sample and mixed. The samples were loaded and electrophoresis was carried out. The gel was removed and placed on a UV transilluminator to visualize bands. Gels were photographed using the Gene Genius Bio imaging system (Syngene).

2.2.10.8 Purification of DNA fragments

After agarose gel electrophoresis, the DNA fragment of interest was excised from the gel and purified using NucleoSpin Extract II Kits (Macherey-Nagel) according to the manufacturer's instructions. Briefly, the DNA was bound in the presence of chaotropic salt to a silica membrane. The binding mixture was loaded directly onto NucleoSpin Extract II columns. Contaminants such as salts and soluble macromolecular components were removed by a washing step with ethanolic buffer NT3. Pure DNA was finally eluted under low ionic strength conditions with slightly alkaline buffer NE (5 mM Tris-Cl, pH 8.5).

2.2.10.9 Dephosphorylation of DNA termini

Following digestion with restriction enzyme endonucleases, dephosphorylation buffer was added to the DNA solution to give a final composition of 50 mM Tris-HCl pH 8.5, 0.1 mM EDTA. One unit of calf intestinal alkaline phosphatase (CIP; Roche) per 2 pmol of DNA ends was added, and then the mixture incubated for 3 minutes at 37°C. An equal amount of CIP was added and incubation at 37°C continued for a further 30 minutes. The reaction was terminated by freezing the solution to -20 °C. The digested DNA was then subjected to agarose gel electrophoresis and purified using illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE HealthCare, UK) according to manufacturer's protocols. The DNA was then ready for ligation.

2.2.10.10 DNA ligation

Generally, a 5-fold molar excess of insert DNA to vector DNA was used in cohesive-end ligation reactions. Ligation reactions were carried out in a 10 µl volume using a rapid ligation kit (Promega). The rapid ligation kit was used according to manufacturer's instructions, with ligation reactions containing 3 Weiss units of T4 DNA ligase (Promega) incubated at 20-25 °C for between 1 and 3 hours before transformation into TOP10F' competent cells.

2.2.10.11 Transformation

Transformations were carried out using chemically-competent cells. The entire ligation was added to 200µl of competent cells and incubated on ice for 30 minutes followed by a heat shock step at 47 °C for 90 seconds. The transformation mixture was then transferred to ice for a further 5 minutes. 500µl of LB was added to the mix and incubated at 37 °C for 30 minutes. The cells were then plated on an LB agar plate containing 100µg/ml ampicillin and incubated overnight at 37 °C.

2.2.10.12 Clone Screen PCR

Colonies were picked and transferred to 20 µl of milliQ water with a sterile toothpick and the suspensions boiled for 5 minutes to lyse the bacterial cells. Each colony was also used to inoculate a numbered grid on an LB agar plate containing 50-100 µg/ml ampicillin. 3 µl of lysate was amplified in a 20 µl reaction containing 10 pmol of each of two flanking primers (sense primer on the vector sequence; anti-sense primer on the inserted DNA), 2 mM dNTPs, 25 mM $MgCL_2$, Perkin Elmer buffer II, 0.1 units of AmpliTaq DNA polymerase. The PCR reaction was performed in a Thermal cycler PTC-200 (MJ Research) using the following conditions: denaturation at 94 °C for 15 seconds, annealing at 53 °C for 15 seconds, and extension at 72 °C for 1 minute/kilobase of predicted product fragment. This cycling was repeated 35 times, and at the end of the procedure, the PCR products were analyzed by agarose gel electrophoresis.

2.2.10.13 Templiphi

Templiphi can be used as a faster and easier alternative to DNA mini-preps for screening colonies when quite large numbers are involved, for example analysis of splice variants and checking for successful mutants in mutagenesis experiments. The reactions were performed using illustra™ TempliPhi 500 amplification kit according to the manufacturer's protocol (GE Healthcare UK limited, UK). Briefly, 2µl of sample buffer was dispensed into PCR tubes. The sample buffer contains random hexamers that prime DNA synthesis nonspecifically and is used to resuspend bacterial cells from a colony or cell culture or any other input DNA. A pin was stuck lightly into the colony to be tested, then put into the sample buffer. After all the colonies were picked, the pins were removed and the samples were heated at 95 °C for 3 minutes in a PCR machine. A master mix containing 2.0µl reaction buffer (salts and deoxynucleotides) and 0.08µl

enzyme mix (ø29 DNA polymerase and random hexamers in 50% glycerol) per reaction was prepared. 2.0µl of master mix was added to the cooled sample tubes and the reactions were incubated at 30 ºC overnight in the PCR machine. The DNA was then used directly for sequencing.

2.2.11 DNA Sequencing

The sequencing reaction contained: Big Dye terminator mix, 1.0µl; 5x Buffer, 1.5µl; primer (1pm/µl), 1.6µl; template + H_2O, 5.9µl in a 10 µl volume. Reactions were performed in a thermal cycler (Bio Rad) using 25 cycles of 96 ºC for 20 seconds, 50 ºC for 10 seconds and 60 seconds for 4 minutes. This was followed by a 4 seconds holding step. DNA reaction products were precipitated by the addition of 5µl 125mM EDTA, 60µl 100% EtOH, and 10µl H_2O. Samples were centrifuged at 14000 rpm for 20 minutes and the supernatant was discarded. The pellets were washed with 70% ethanol to carefully remove all supernatant and air dried for around 5 minutes. DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system following the manufacturer's instructions with analysis using an ABI 3730 sequencer. The sequencer was operated by the Biomolecular Resource Facility, John Curtin School of Medical Research at ANU.

2.2.12 Statistical analysis

A student's two-tailed non-paired *t*-test was used to determine the statistical significance.

Chapter 3

IL-3-driven differentiation of NB4 promyelocytic leukemia cells

3.1 Introduction

The IL-3-driven myeloid differentiation pathway, which is the focus of the work described in this thesis, has so far received little attention and remains poorly understood. The properties of cytokine-deficient mice have shown that hematopoiesis driven by IL-3, GM-CSF and IL-5 (de Groot *et al.,* 1998) can be disrupted with minimal effects on steady-state blood cell formation. Thus steady state hematopoiesis and cytokine-driven hematopoiesis involve separate mechanisms. As previously discussed in Chapter 1, the concept of two differentiation pathways is also supported by studies of progenitors from C/EBPα $^{-/-}$ mice (Zhang *et al.,* 2002). In terms of cytokine-driven differentiation, there remains controversy as to whether cytokine signaling has an instructive role or whether cytokines merely provide support for stochastic differentiation processes (Robb, 2007).

Studies on IL-3Rα indicate that it is a marker of leukemic stem cells and suggest that it may be involved in the pathogenesis of AML. However, its role, if any, is not currently understood. IL-3Rα (CD123) is over-expressed in 45% of AML patients (Graf *et al.,* 2004), 40% of B- lineage ALL patients (de Waele *et al.,* 2001) and 70% of immature T-lineage ALL patients (Lhermitte *et al.,* 2006). The elevated expression of CD123 in acute leukemia is associated with enhanced leukemic blast cell proliferation, increased cell cycle activity, and poorer prognosis (Testa *et al.,* 2002). Up-regulation of CD123 was also discovered in human acute myelogenous leukemia stem cells (Jordan *et al.,* 2000). New anti-leukemic drugs targeting the IL-3 receptor are now under development (Testa *et al.,* 2004).

Our group has recently identified a new splice variant of IL-3Rα (lL-3Rα SP2) which is active in myeloid differentiation (Chen *et al.,* 2009). Ectopically expressed hIL-3Rα

SP2 plus hβc allowed hIL-3-driven differentiation of multi-potential FDCP-mix and myeloblastic M1 cells (Chen *et al.,* 2009). The expression of hIL-3Rα SP2 was detected in various human leukemia cell lines, including NB4, KGla, THP-1 and MEG-01 cells. However, the levels of the individual hIL-3Rα isoforms in AML patients have not yet been determined.

Analysis of IL-3 binding to AML blasts showed that over 80% had high affinity IL-3 receptors (Alexandera *et al.,* 2001) even though IL-3 did not induce differentiation as it does with normal myeloid progenitors. Also, a naturally occurring isoform of hβc ($h\beta_{IT}$) which possesses a truncated defective cytoplasmic region has been identified. Increased expression of β_{IT} was found in the majority of AML patients tested (Gale *et al.,* 1998). Collectively, this data suggests that AML blasts are commonly blocked in both the steady state and IL-3-induced myeloid differentiation pathways.

The leukemic cell line, NB4 isolated from a promyelocytic leukemia patient (Lanotte *et al.,* 1991), has been studied extensively because it can be differentiated along the granulocytic or monocyte/macrophage pathways by various differentiation inducers including ATRA (Tohda *et al.,* 1992; Clark *et al.,* 2004). Acute promyelocytic leukemia is characterized by maturation arrest at the promyelocytic stage of development caused by a novel fusion protein resulting from the reciprocal translocation involving the retinoic acid receptor alpha (RARα) on chromosome 17 with the promyelocytic leukemia gene (PML) on chromosome 15 (Melnick and Licht, 1999). The PML-RARα fusion protein blocks steady state myeloid differentiation (Benoit *et al.,* 2001). NB4 also does not differentiate in response to hIL-3 or hGM-CSF (Hsu *et al.,* 1996) indicating that the cytokine-induced differentiation pathway is blocked in these cells. However, interestingly, the combined neutralization of hIL-3, hGM-CSF and hG-CSF inhibits *all-trans* retinoic acid (ATRA)-induced differentiation of NB4 (Matsui *et al.,* 2005)

suggesting the possibility that IL-3 and/or GM-CSF signaling might be involved in the ATRA-induced differentiation pathway.

In the results presented in this Chapter, the IL-3 receptor system in NB4 promyelocytic leukemia cells is studied, hIL-3-driven differentiation is partially restored by ectopic expression of the receptor and the possible involvement of the IL-3 receptor system in ATRA-induced differentiation is investigated.

3.2 Results

The human IL-3 receptor system is composed of an IL-3 receptor α subunit (hIL-3Rα; CD123) and the βc subunit (CD131). The βc subunit is shared with IL-5 and GM-CSF receptors. hβc cannot detectably bind any of the three cytokines directly but complexes with the respective α subunits and the ligands to form high-affinity receptors. hβc is thought to be responsible for the majority of the signaling through the IL-3/IL-5/GM-CSF receptors (Hayashida *et al.,* 1990; Kitamura *et al.,* 1991; Tavernier *et al.,* 1991; Takaki *et al.,* 1993). The hIL-3R system was investigated in NB4 promyelocytic leukemia cells.

3.2.1 The common beta-associated protein (CBAP)

A potentially important new lead relevant to βc function in growth and differentiation is the recent identification of a novel transmembrane protein, common β-chain associated protein (CBAP) (Kao *et al.,* 2008), which can induce apoptosis in factor-dependent cells via a mitochondrial pathway. Its expression in NB4 cells was investigated as it might potentially be relevant to myeloid differentiation.

3.2.1.1 Expression of CBAP in NB4 cells undergoing differentiation

CBAP mRNA levels were determined before and after induction of differentiation. NB4 cells were treated with ATRA for different times as indicated in Figure 3.1. The expression of human CBAP mRNA was increased only very slightly in NB4 cells induced by ATRA (Fig.3.1), but was clearly expressed in un-induced cells. The expression of CBAP in NB4 cells means it could potentially play a role in myeloid differentiation.

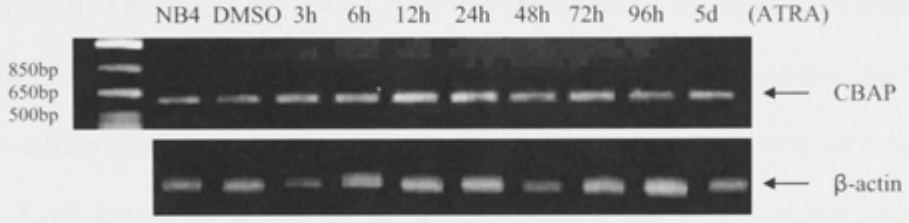


Figure 3.1: The levels of hCBAP mRNA in NB4 cells before and after induction of differentiation by ATRA was measured by RT-PCR as described in Chapter 2. ATRA was dissolved in DMSO and included in the medium at a final concentration of 1μM. NB4, indicates untreated cells; DMSO, indicates cells treated with DMSO alone; the times of induction are indicated in the figure. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

3.2.1.2 Expression of CBAP in mouse cell lines undergoing IL-3-dependent growth or differentiation

To further test for a possible role in growth or differentiation, the levels of CBAP mRNA were tested in a number of mouse cell lines. Some were undergoing mIL-3-dependent growth and others undergoing hIL-3-induced differentiation enabled by ectopic expression of the hIL-3R. Surprisingly, the expression of CBAP mRNA was detected only in WEHI-3BD+ cells (Fig.3.2). This sub-line is capable of differentiation in G-CSF (Wu *et al.,* 1989), but was grown in the absence of cytokine in this experiment. The sequence of the CBAP cDNA isolated from these cells confirmed its identity (see below) and the validity of this analysis. The results indicate that CBAP is not required for hIL-3-driven differentiation or mIL-3-driven growth of a number of mouse cell lines. The role of CBAP in IL-3-induced differentiation was therefore not investigated further.

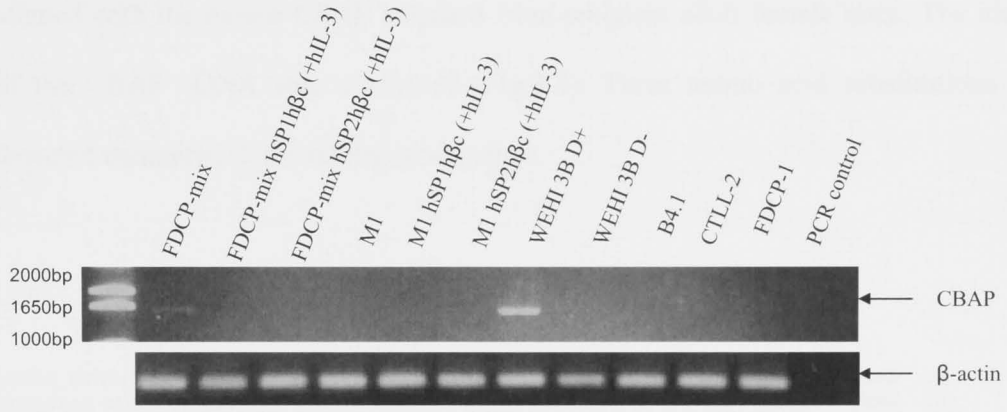


Figure 3.2: The levels of CBAP mRNA in mouse cell lines measured by RT-PCR. FDCP-mix, B4.1, FDCP1 were undergoing IL-3-dependent growth and CTLL-2 was undergoing IL-2-dependent growth. FDCP-mix and M1 cells ectopically expressing hIL-3Rα SP2/hβc were undergoing hIL-3-driven differentiation. RNase-free water was used as PCR control. Results represent one of at least two independent experiments.

3.2.1.3 Sequence of CBAP from WEHI-3B D+

Expression of CBAP mRNA was only detected in the WEHI-3B D+ cell line. WEHI-3B D+ is a leukemic cell line which constitutively expresses mIL-3 and will differentiate in response to G-CSF (Li *et al.,* 1993). The full length mCBAP cDNA from WEHI-3B D+ cell line was isolated and sequenced. The amino acid translation is shown in Figure 3.3 aligned with the mouse CBAP standard from pregnant adult female mice. The identity of the CBAP cDNA was confirmed (Fig.3.3). Three amino acid substitutions were detected compared to the reference sequence.

```
mCBAP_WEHI-3BD+          MASTVWGGAPWWGPPPPAPARPLTDIDFCSGAQLQELTQLIQELGVQESW  50
Standard mouse mCBAP     MASTVWGGAPWWGPPPPAPARPLTDIDFCSGAQLQELTQLIQELRVQESW  50

mCBAP_WEHI-3BD+          SEGPKPGADLLRAKDFVFALLGLVHRQDPRFPPQAELLLLRGGIREGSLD 100
Standard mouse mCBAP     SEGPKPGADLLRAEDFVFALLGLVHRQDPRFPPQAELVLLRGGIREGSLD 100

mCBAP_WEHI-3BD+          LGHAPLGPYSRGPHYDAGFTLLVPVFSLDGTGPELLLDLESCSAWLRLPE 150
Standard mouse mCBAP     LGHAPLGPYSRGPHYDAGFTLLVPVFSLDGTGPELLLDLESCSAWLRLPE 150

mCBAP_WEHI-3BD+          LMRGILVREAWQDCLGPPVPEESDMTHQTHSKESPTDRENSVDPSHDYVP 200
Standard mouse mCBAP     LMRGILVREAWQDCLGPPVPEESDMTHQTHSKESPTDRENSVDPSHDYVP 200

mCBAP_WEHI-3BD+          EPEPHMSLQKSSSDLSESQSSYKDITNPETPEPLETLSSDALDADESQVP 250
Standard mouse mCBAP     EPEPHMSLQKSSSDLSESQSSYKDITNPETPEPLETLSSDALDADESQVP 250
```

Figure 3.3: Alignment of amino acid sequences of mouse CBAP derived from WEHI-3B D+ cells with the reference CBAP sequence (Kao *et al.,* 2008). The full length mCBAP cDNA from WEHI-3B D+ cell line was isolated and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

3.2.2 Expression of hβc, hIL-3Rα SP1 and hGM-CSFRα in NB4 cells undergoing differentiation

NB4 cells can differentiate to neutrophils in the presence of ATRA (*all-trans* retinoic acid) but do not differentiate in response to hIL-3 or hGM-CSF. RT-PCR was used to investigate the levels of expression of endogenous hIL-3Rα SP1, hGM-CSFRα and hβc mRNAs in NB4 cells before and after induction of differentiation with ATRA. All were readily detectable in un-induced NB4 cells. The expression of hβc was found to be increased significantly in NB4 cells undergoing differentiation induced by ATRA. The expression levels of IL-3Rα SP1 and GM-CSFRα showed no significant change in NB4 cells with or without ATRA (Fig.3.4).

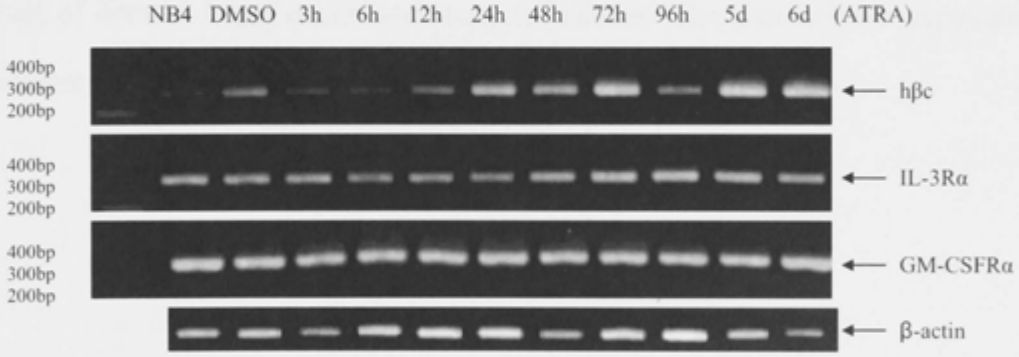


Figure 3.4: The levels of IL-3Rα SP1, GM-CSFRα and hβc mRNA in NB4 cells before and after induction of differentiation by ATRA was measured by RT-PCR. ATRA was dissolved in DMSO and included in the medium at a final concentration of 1μM. NB4, indicates untreated cells; DMSO, indicates cells treated with DMSO alone; the times of induction are indicated in the figure. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

3.2.3 DNA sequences of endogenous hIL-3 receptor subunits in NB4 cells

Since hIL-3 does not induce differentiation of NB4, in contrast to its action on normal myeloid progenitors, it was of interest to verify the sequence of the endogenous IL-3 Rα mRNAs to verify the absence of mutations which might affect function. Sequence analysis of full length hIL-3Rα cDNA clones isolated from NB4 cells showed the presence of approximately equal numbers of clones of the IL-3Rα SP1 and IL-3Rα SP2 (211-444 base deletion) isoforms before and after treatment with ATRA. These clones had no amino acid substitutions compared with the reference sequence (Chen *et al.*, 2009). Also detected was a cDNA with a 211-329 base deletion (Fig.3.5). This clone is missing the first exon encoding domain 1 which results in the deletion of approximately half of domain 1 and causes premature termination. The data indicate expression of normal mRNAs encoding both hIL-3Rα isoforms in NB4 cells.

```
Single exon deletion    MVLLWLTLLLIALPCLLQTKE----------------------------  21
hIL-3Rα SP2             MVLLWLTLLLIALPCLLQTKE----------------------------  21
hIL-3Rα SP1             MVLLWLTLLLIALPCLLQTKEDPNPPITNLRMKAKAQQLTWDLNRNVTDI  50
Wild type hIL-3Rα       MVLLWLTLLLIALPCLLQTKEDPNPPITNLRMKAKAQQLTWDLNRNVTDI  50

Single exon deletion    -----------SEQ·                                     24
hIL-3Rα SP2             ------------------------------------------------S  22
hIL-3Rα SP1             ECVKDADYSMPAVNNSYCQFGAISLCEVTNYTVRVANPPFSTWILFPENS 100
Wild type hIL-3Rα       ECVKDADYSMPAVNNSYCQFGAISLCEVTNYTVRVANPPFSTWILFPENS 100

hIL-3Rα SP2             GKPWAGAENLTCWIHDVDFLSCSWAVGPGAPADVQYDLYLNVANRRQQYE  72
hIL-3Rα SP1             GKPWAGAENLTCWIHDVDFLSCSWAVGPGAPADVQYDLYLNVANRRQQYE 150
Wild type hIL-3Rα       GKPWAGAENLTCWIHDVDFLSCSWAVGPGAPADVQYDLYLNVANRRQQYE 150

hIL-3Rα SP2             CLHYKTDAQGTRIGCRFDDISRLSSGSQSSHILVRGRSAAFGIPCTDKFV 122
hIL-3Rα SP1             CLHYKTDAQGTRIGCRFDDISRLSSGSQSSHILVRGRSAAFGIPCTDKFV 200
Wild type hIL-3Rα       CLHYKTDAQGTRIGCRFDDISRLSSGSQSSHILVRGRSAAFGIPCTDKFV 200

hIL-3Rα SP2             VFSQIEILTPPNMTAKCNKTHSFMHWKMRSHFNRKFRYELQIQKRMQPVI 172
hIL-3Rα SP1             VFSQIEILTPPNMTAKCNKTHSFMHWKMRSHFNRKFRYELQIQKRMQPVI 250
Wild type hIL-3Rα       VFSQIEILTPPNMTAKCNKTHSFMHWKMRSHFNRKFRYELQIQKRMQPVI 250
```

Figure 3.5: Alignment of amino acid sequences of hIL-3Rα derived from NB4 cells before and after treatment with ATRA. The full length hIL-3Rα cDNA clones were isolated from NB4 cells and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

Previous work by others in our group had shown that the hβc mRNAs expressed by NB4 cells were largely abnormal. Using flanking primers, seven clones were obtained and sequenced. Only one normal cDNA clone out of seven was obtained, with the rest having deletions (Y. Wu, J. Chen and I. Young, unpublished). After induction of differentiation with ATRA, six out of eight clones were normal. This suggests that a low level of normal hβc mRNA is present in NB4 cells prior to induction with ATRA.

3.2.4 Protein expression levels of hIL-3 receptor subunits in NB4 cells

The endogenous levels of hIL-3Rα and hβc were measured by flow cytometry. The levels of hIL-3Rα and hβc were detectable but very low in NB4 parental cells. The endogenous expression levels of hIL-3Rα and hβc in NB4 cells did not change significantly after NB4 cells were treated with ATRA (Fig.3.6). The antibody used detects both IL-3Rα SP1 and SP2 so the levels of the individual isoforms could not be established by flow cytometry. However, our group has previously measured the expression of both isoforms in NB4 by Western blotting (Chen *et al.*, 2009) and shown that SP2 protein is expressed in NB4 cells together with the SP1 protein which is the major isoform.

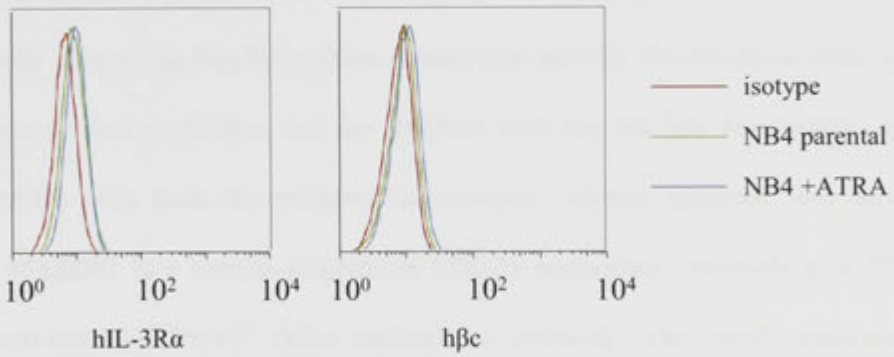


Figure 3.6: The levels of hIL-3Rα and hβc endogenous expression in NB4 cells were measured by FACS. The red line histograms denote non-specific fluorescence obtained with the isotype control antibodies and are overlaid with the green and blue line histograms obtained after labeling with both the primary anti-receptor subunit antibody and secondary reagent. MAB301 is a mouse anti-human IL-3Rα (CD123) monoclonal antibody and 3D7 is a mouse anti-human hβc (CDw131) monoclonal antibody. Results represent one of at least two independent experiments.

3.2.5 Ectopic expression of hIL-3Rα and hβc subunits in NB4 cells

Since the endogenous levels of the hIL-3R subunits were very low in NB4 cells and there was some doubt about the amount of fully functional hβc, it was of interest to increase the levels of expression of the IL-3R. cDNAs encoding human IL-3Rα SP1 and SP2 were cloned into the pEFIRES-N (G418) expression vector and a cDNA encoding hβc cloned into pEFIRES-P (puromycin). Double stable transfections of expression constructs into NB4 cells were carried out and selection made using G418 and/or puromycin. After the stable transfectants were recovered, the levels of ectopically expressed hIL-3Rα SP1 or SP2 and hβc in NB4 cells were measured by flow cytometry. Ectopic receptor expression was confirmed using a 2-step antibody labeling procedure. Dual transfectants expressing both the hIL-3Rα together with hβc were assessed by using 2-color analysis with FITC- and PE-conjugated secondary antibodies, respectively. The green line histograms denote non-specific fluorescence obtained with the isotype control antibodies and are overlaid with the red line histograms obtained after labeling with both the primary anti-receptor subunit antibody and secondary reagent. MAB301 is a mouse anti-human CD123 monoclonal antibody and 3D7 is a mouse anti-human CDw131 (hβc) monoclonal antibody. The results indicate good expression of the hIL-3Rα and hβc subunits (Fig.3.7). The levels of the receptors were considerably higher than the endogenous levels (see above).

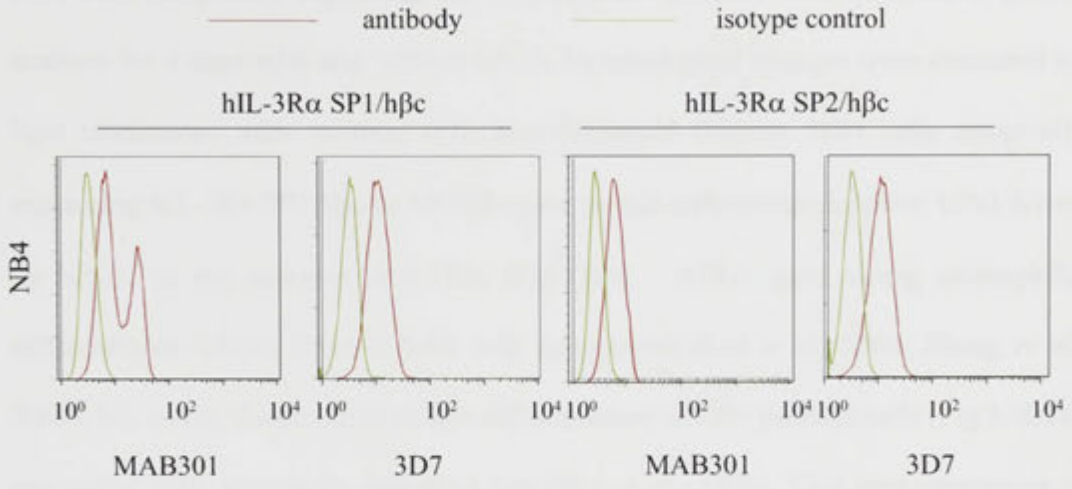


Figure 3.7: The levels of ectopically expressed hIL-3Rα SP1 or SP2 and hβc in NB4 cells were measured by FACS. Dual transfectants expressing both the hIL-3Rα together with hβc were assessed by using 2-color analysis with FITC- and PE-conjugated secondary antibodies, respectively. The green line histograms denote non-specific fluorescence obtained with the isotype control antibodies and are overlaid with the red line histograms obtained after labeling with both the primary anti-receptor subunit antibody and secondary reagent. MAB301 is a mouse anti-human IL-3Rα (CD123) monoclonal antibody and 3D7 is a mouse anti-human hβc (CDw131) monoclonal antibody. Results represent one of at least two independent experiments.

3.2.6 Differentiation of NB4 cells ectopically expressing hIL-3Rs in response to hIL-3

NB4 cells ectopically expressing the two different hIL-3Rs were cultured in growth medium for 4 days with and without hIL-3. Morphological changes were examined by light microscopy after staining with May-Grunwald Giemsa. NB4 cells ectopically expressing hIL-3Rα SP1/hβc or SP2/hβc gave partial differentiation (about 15%) driven by hIL-3, in the absence of ATRA (Fig.3.8A). ATRA gave strong neutrophilic differentiation (about 70%) of NB4 cells as expected (Lee *et al.,* 2002, Zhang *et al.,* 2008). hIL-3 was shown not to induce differentiation of NB4 parental cells (Fig.3.8B) in agreement with previously published data (Hsu *et al.,* 1996). Thus over-expression of the IL-3R system resulted in a partial bypass of the differentiation block in NB4 cells.

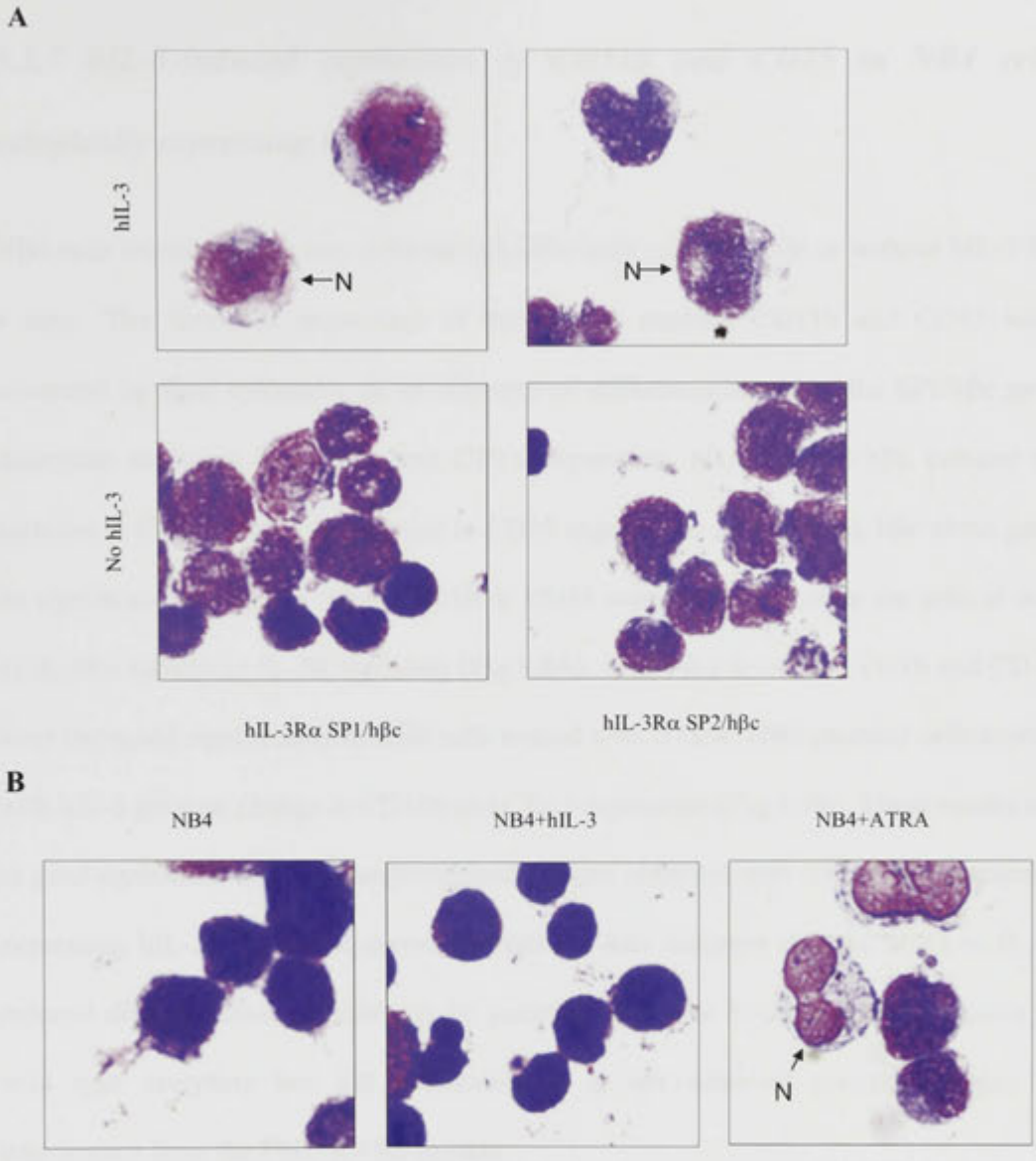


Figure 3.8: Morphological changes in **A:** NB4 cells expressing the hIL-3R system after 4 days culture with hIL-3 (220U/ml); **B:** NB4 parental cells plus ATRA (1μM) or hIL-3 (220U/ml). Morphological examination was made by light microscopy after May-Grunwald-Giemsa staining. NB4 cells ectopically expressing hIL-3Rα SP1/hβc or SP2/hβc gave partial differentiation to neutrophil-like cells driven by hIL-3 and ATRA gave strong neutrophilic differentiation of NB4 cells. *N*, neutrophil. Scale bar = 10 μm. Results represent one of three independent experiments.

3.2.7 hIL-3-induced expression of CD11b and CD15 in NB4 cells ectopically expressing hIL-3Rs

NB4 cells expressing the two different hIL-3Rs were cultured with or without hIL-3 for 4 days. The levels of expression of the surface markers CD11b and CD15 were measured by flow cytometry as an indicator of differentiation. hIL-3Rα SP2/hβc gave detectable increases in CD11b and CD15 expression. hIL-3Rα SP1/hβc induced an increase in CD11b but little increase in CD15 expression. As expected, hβc alone gave no significant increase in either CD11b or CD15 expression indicating the critical role of IL-3Rα subunit in IL-3R signaling (Fig.3.9A). While the levels of CD11b and CD15 were increased significantly in NB4 cells treated with ATRA, NB4 parental cells treated with hIL-3 gave no change in CD11b and CD15 expression (Fig.3.9B). These results are in good agreement with the morphological changes observed with NB4 cells ectopically expressing hIL-3R described above. Overall the data indicates that the block in IL-3-induced differentiation of NB4 can be partially overcome by increased expression of wild type receptors but full differentiation is not achieved presumably due to interference from the PML-RARα protein.

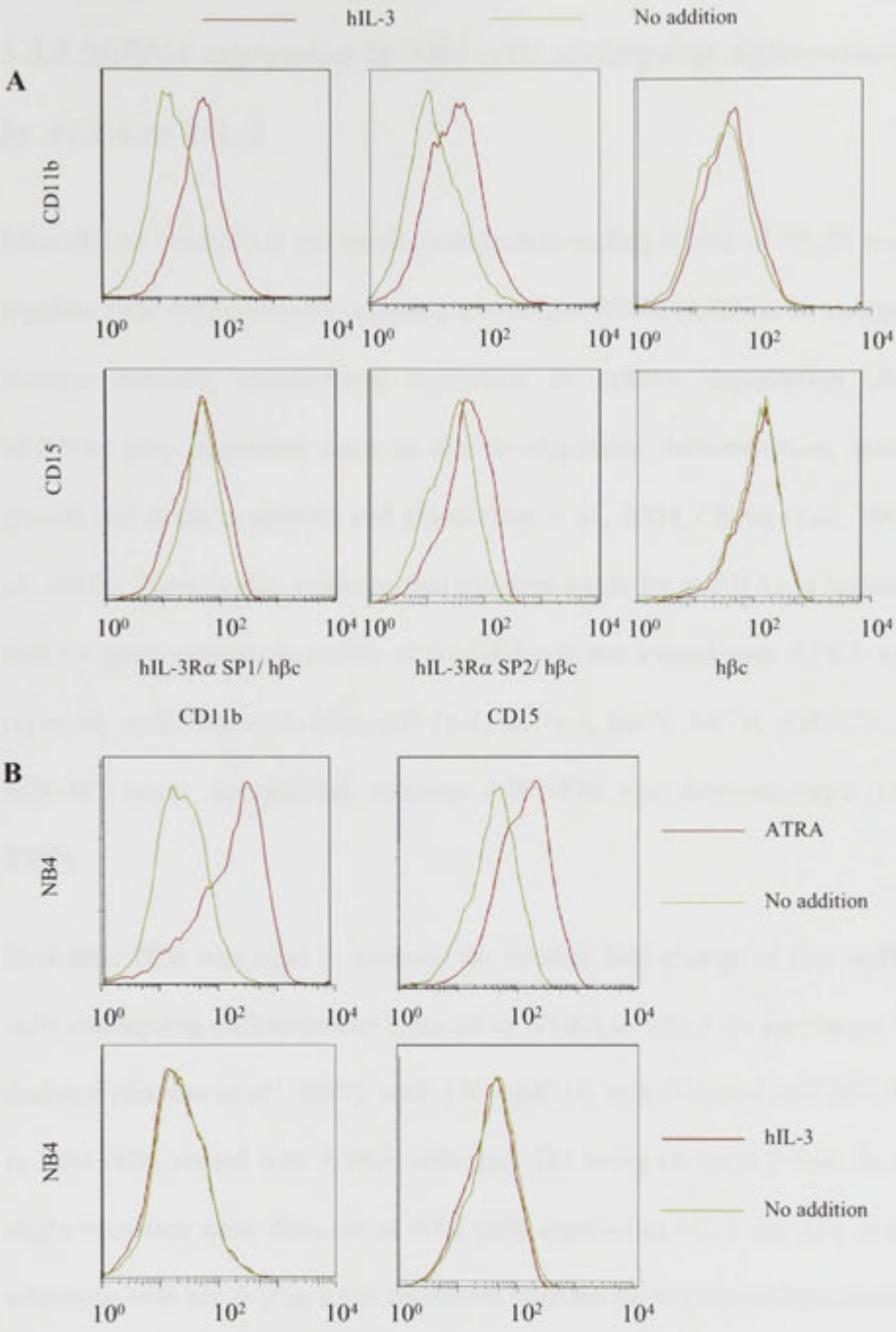


Figure 3.9: FACS analysis of expression of the surface markers CD11b and CD15. **A:** NB4 cells ectopically expressing the hIL-3R system were cultured with or without hIL-3 (220U/ml) for 4 days. Expression of CD11b and CD15 in NB4 cells was measured by FACS. **B:** FACS analysis of expression of CD11b and CD15 in parental NB4 cells treated with ATRA (1μM) or hIL-3 (220U/ml). Results represent one of three independent experiments.

3.2.8 MiRNA expression in NB4 cells undergoing differentiation induced by ATRA or hIL-3

MicroRNAs (miRNAs) are small, non-protein-coding RNAs of 19–25 nucleotides that regulate gene expression by targeting messenger RNAs (mRNA) in a sequence-specific manner inducing translational repression or mRNA degradation (Bartel, 2004). MiRNAs play important roles in the development, differentiation, metabolism, cell growth and death in animals and plants (Poy *et al.,* 2004; Cheng *et al.,* 2005; Dresios *et al.,* 2005). There is also evidence that supports a role for miRNAs in hematopoiesis. The miRNA gene expression profile of the NB4 cell line treated with ATRA was previously reported. miR-15a, miR-15b, miR-16-1, let-7a-3, let-7c, let-7d, miR-223, miR-342 and miR-107 were upregulated, whereas miR-181b was downregulated (Garzon *et al.,* 2007).

Real-time PCR was used to measure the relative fold change of four miRNAs in NB4 cells undergoing differentiation induced by ATRA or hIL-3. In agreement with previous findings (Garzon *et al.,* 2007), miR-15a, miR-16, miR-223 and miR-342 were elevated in NB4 cells treated with ATRA with miR-223 being elevated 5-fold. In contrast, only slight increases were detected in NB4 cells expressing hIL-3 Rα SP1 or SP2/hβc after treatment with hIL-3 (Fig.3.10) consistent with the incomplete differentiation observed.

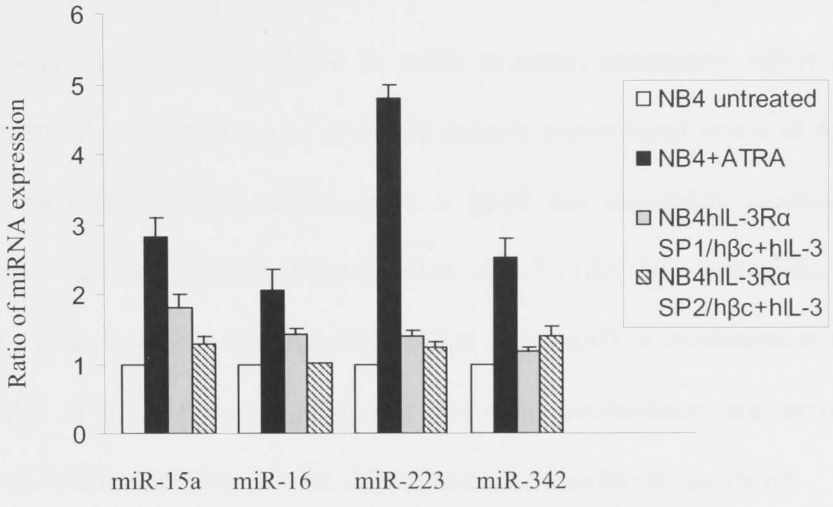


Figure 3.10: Relative fold change of four miRNAs in NB4 cells undergoing differentiation induced by ATRA or hIL-3 was measured by real-time RT-PCR. NB4 cells expressing the hIL-3R system were cultured with hIL-3 for 4 days. And NB4 parental cells were treated with or without ATRA for 4 days. Expression of each component was presented as the fold-expression ratio of the level in differentiated cells over the level in NB4 untreated cells. U6B small nuclear RNA was used as reference gene. Results were verified in at least three independent experiments.

3.2.9 Investigation of possible synergistic effects between hIL-3 or hGM-CSF and ATRA in the induction of differentiation

The experiments described above showed that over-expression of the hIL-3R partially overcame the block in differentiation in NB4 cells. It was of interest to see if any synergism could be detected between IL-3 or GM-CSF signaling through their endogenous receptors and ATRA. In order to detect synergistic effects, preliminary experiments were carried out to establish suitable sub-optimal levels of ATRA. It was established that 0.005μM ATRA gave a small but detectable increase in CD11b expression indicating slight differentiation (Fig.3.11A). No synergistic increases in CD11b expression were detected with ATRA (0.005μM) in combination with hIL-3 or hGM-CSF (Fig.3.11B) indicating that separate mechanisms are involved in the induction of differentiation via the ATRA and cytokine-driven pathways.

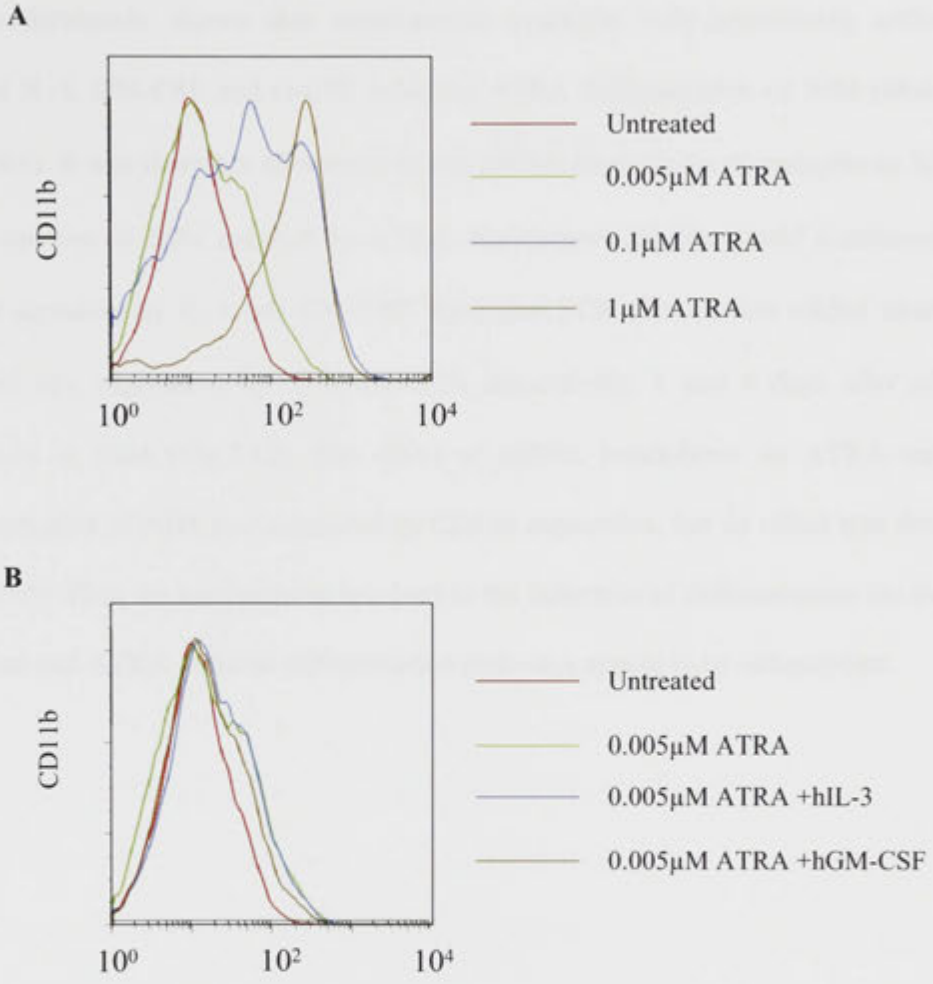


Figure 3.11: Investigation of possible synergistic effects between hIL-3 or hGM-CSF and sub-optimal levels of ATRA. **A:** NB4 cells were treated with different concentrations of ATRA (0.005μM, 0.1 μM, 1 μM) for 4 days and the levels of CD11b expression determined by FACS. **B:** NB4 cells were treated with a sub-optimal level of ATRA (0.005μM) and hIL-3 (220U/ml) or hGM-CSF (290U/ml) for 4 days and CD11b expression measured by FACS. Results represent one of three independent experiments.

3.2.10 Effect of siRNA knockdown of hβc on ATRA-induced NB4 differentiation

It was previously shown that simultaneous treatment with neutralizing antibodies against IL-3, GM-CSF and G-CSF inhibited ATRA differentiation of NB4 (Matsui *et al.*, 2005). It was therefore of interest to test siRNA knockdown of endogenous hβc on differentiation of NB4 induced by ATRA. Knockdown of hβc would simultaneously inhibit signaling by IL-3 and GM-CSF. Real-time PCR showed that siRNA treatment reduced hβc expression by 90% and 75% respectively, 1 and 4 days after siRNA treatment in NB4 (Fig.3.12). The effect of siRNA knockdown on ATRA induced differentiation of NB4 was measured by CD11b expression, but no effect was detected (Fig.3.13). Thus the mechanisms involved in the induction of differentiation via the IL-3-driven and ATRA-induced differentiation pathways appear to be independent.

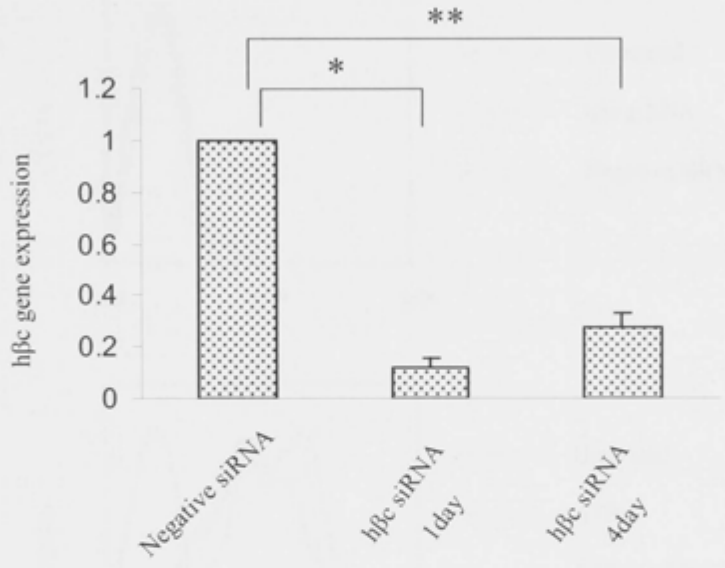


Figure 3.12: siRNA knockdown of hβc mRNA in NB4 cells by hβc siRNA. NB4 cells were treated with hβc siRNA and a negative siRNA control. The levels of hβc mRNA in NB4 cells treated with hβc siRNA for 1 and 4 days was measured by real-time RT-PCR and compared with NB4 cells treated with negative siRNA control. Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.001$; **, $p<0.01$ by student's *t* test.

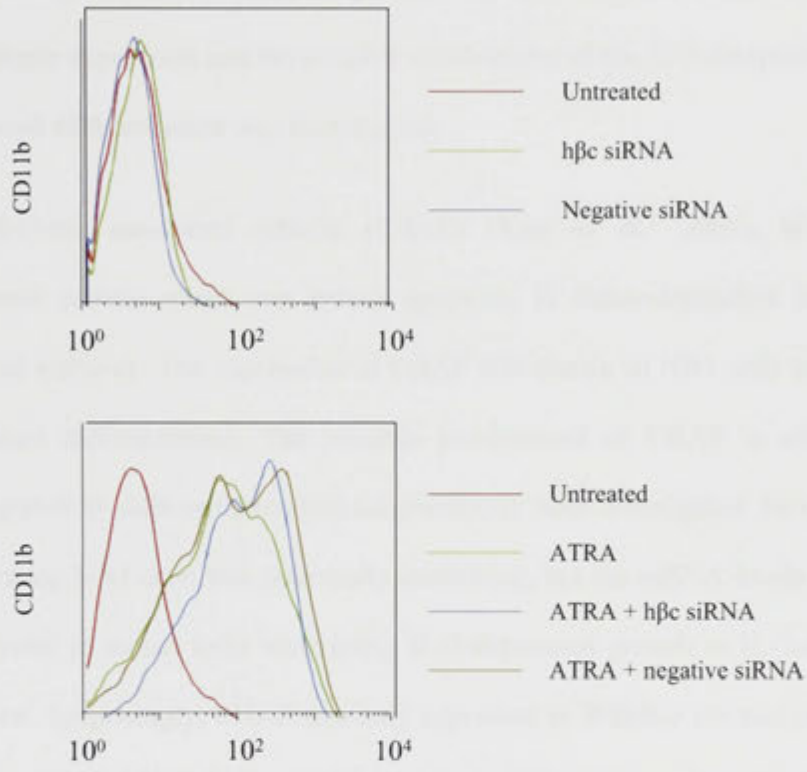


Figure 3.13: The effect of hβc siRNA knockdown on ATRA induced differentiation of NB4 was measured by CD11b expression. NB4 cells were treated with 1 μM ATRA and/or hβc siRNA or negative siRNA control for 4 days. Expression of CD11b in NB4 cells was measured by FACS. Results represent one of three independent experiments.

3.3 Discussion

In the results described in this Chapter, the hIL-3R system was investigated in NB4 cells, hIL-3-driven differentiation was partially restored after elevation of the IL-3R levels by ectopic expression and the possible involvement of the IL-3 receptor system in ATRA-induced differentiation was investigated.

Common β-chain associated protein (CBAP) (Kao *et al.*, 2008), is a novel transmembrane protein which can induce apoptosis in factor-dependent cells via a mitochondrial pathway. The expression of CBAP was shown in NB4 cells undergoing ATRA-induced differentiation. The possible involvement of CBAP in either IL-3-dependent growth or differentiation had not previously been investigated. Its expression in differentiating NB4 cells was potentially interesting, but the mRNA levels of CBAP were also tested in mouse cells undergoing IL-3-dependent growth or IL-3-dependent differentiation. Surprisingly, CBAP was only expressed in WEHI-3 D+ and was absent in all strains undergoing IL-3-dependent growth or differentiation. It is therefore clear that CBAP does not play a critical role in IL-3-dependent growth or differentiation in mouse cells and it is reasonable to conclude that it would also not play a critical role in IL-3-driven differentiation of human myeloid cells.

The IL-3R system was investigated in NB4 cells. It was shown by DNA sequencing that the sequences of the endogenous IL-3Rα isoforms were normal. Previous work in our group had shown by Western blotting that normal levels of protein of the two isoforms are expressed in NB4 cells (Chen *et al.*, 2009). Flow cytometry demonstrated low but detectable levels of IL-3Rα and hβc in NB4 cells. The levels of the IL-3R were considerably elevated by ectopic expression. Interestingly, hIL-3-driven differentiation was restored in NB4 cells ectopically expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc

as shown by morphological analysis and surface marker expression but the differentiation was only partial. This indicates that the block in IL-3-induced differentiation of NB4 can be partially overcome by increased expression of wild type IL-3 receptors. However, full differentiation could not be restored, probably due to the dominant negative effects of the PML-RARα fusion protein, which are only fully overcome by ATRA.

MiRNAs play important roles in the differentiation, development, cell growth and hematopoiesis (Cheng *et al.,* 2005; Dresios *et al.,* 2005). It has been reported that miR-223 is a key member of a regulatory circuit involving C/EBPα and NFI-A that control granulocytic differentiation in ATRA-treated APL cell lines (Fazi *et al.,* 2005). In agreement with previous findings (Garzon *et al.,* 2007), miR-15a, miR-16, miR-223 and miR-342 were elevated in NB4 cells treated with ATRA with miR-223 levels being elevated 5-fold. Only slight increases were detected in NB4 cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc after treatment with hIL-3. The minor increases in miRNAs are in agreement with the partial differentiation of NB4 cells ectopically expressing the two hIL-3Rs induced by hIL-3.

It has been shown that neutralizing antibodies to hIL-3, hGM-CSF and hG-CSF partially block ATRA-induced differentiation of NB4 suggesting a possible involvement of βc receptor signaling in the ATRA-induced differentiation pathway (Matsui *et al.,* 2005). However no synergistic effects between hIL-3 or hGM-CSF and ATRA were detected. Similarly, siRNA knockdown of hβc had no significant effect on NB4 differentiation induced by ATRA. Collectively these results indicate that the mechanisms involved in induction of differentiation via the IL-3-driven and ATRA-induced differentiation pathways are independent in NB4 cells.

Chapter 4

Role of CD44 and osteopontin in myeloid differentiation

4.1 Introduction

A major goal of AML treatment is the development of differentiation therapies to overcome the differentiation block in leukemia. The clinical use of ATRA to treat PML, particularly in combination with arsenic trioxide and chemotherapy, is the most successful differentiation therapy to date (Petrie *et al.,* 2009). Recently, some exciting new findings on the differentiation of AML blasts and leukemic stem cells were reported which involved ligation of CD44 (Gadhoum *et al.,* 2004). In contrast to ATRA treatment which is limited to PML, CD44 ligation seems to be applicable to most types of AML (Gadhoum *et al.,* 2004) although the efficiency varies with the AML sub-type (Charrad *et al.,* 1999). CD44-induced differentiation was very effective in reducing the levels of PML-RARα protein in AML3 suggesting a possible relationship with ATRA-induced differentiation. Also, G-CSF or M-CSF mRNAs were induced in some cases (Charrad *et al.,* 1999) suggesting possible links with cytokine-induced differentiation. Recently, it was shown that autocrine/paracrine secretion of GM-CSF is required for AML5 blast differentiation by ligated CD44 (Delaunay *et al.,* 2008) suggesting the involvement of the βc receptor system.

CD44 is a cell surface transmembrane glycoprotein which is involved in normal hematopoiesis and in leukemia (Ghaffari *et al.,* 1999). The CD44 gene contains 20 exons, many of which are subject to alternative mRNA splicing resulting in a large number of possible transcripts. The most common form of CD44 is referred to as CD44H (hematopoietic) or CD44s (standard). CD44 variants contain additional exons referred to as variant exons (v1-v10) (Ghaffari *et al.,* 1999). CD44 is a receptor for hyaluronic acid (HA) and is involved in lymphocyte activation, recirculation and homing, adhesion of the extracellular matrix, angiogenesis, cell proliferation, cell

differentiation and cell migration (Adamia *et al.,* 2005). CD44s (Iida and Bourguignon, 1995) is expressed by all types of mature blood cells and the majority of mononuclear bone marrow precursors. The level of its expression varies according to the hematopoietic cell lineage and stage of differentiation. Expression of CD44 variants has been associated with poor prognosis and increased metastatic spread in acute leukemia (Bendall *et al.,* 2000). For example, the expression of CD44v6 (CD44 isoform containing exon v6) has been shown to be increased in ALL (Bendall *et al.,* 2004) and in AML (Legras *et al.,* 1998) and correlates with shorter overall survival.

CD44 is a major receptor for HA, which has been identified as a critical component of the hematopoietic stem cell (HSC) niche (Nilsson *et al.,* 2003). Administration of HA to mice following 5-fluorouracil treatment enhanced the recovery of leucocytes and platelets in the peripheral blood, further suggesting that HA has important roles *in vivo* in HSC regulation (Matrosova *et al.,* 2004).

In primary IL-3-dependent mouse bone marrow cells, expression and activation of CD44 has been shown to be important for the anti-apoptotic activities of IL-3 and GM-CSF (Lin *et al.,* 2000). IL-3 and GM-CSF upregulate the expression of CD44v4 and CD44v6, and the increased CD44 expression is accompanied by elevated expression of IL-3Rα in activated airway eosinophils (Bates *et al.,* 2004). However, the possible relationship of CD44 and the IL-3 receptor system in leukemia remains unclear.

OPN is a secretory extracellular matrix protein that has been implicated in cancer-associated mechanisms such as metastasis, invasion and angiogenesis and is a ligand for CD44. Three OPN isoforms (OPN-a, -b and -c) derived from alternative splicing are known to exist, but their functional specificity remains unclear (Chae *et al.,* 2009). Overexpression of OPN is a feature of multiple myeloma and chronic myeloid leukemia through the regulation by BCR-ABL oncogene. In CML and multiple myeloma patients,

the expression of OPN is involved in the progression and increased aggressiveness of disease (Hickey *et al.*, 2005). OPN-c is a selective marker of breast cancer (Patani *et al.*, 2008). OPN has a well-documented role in bone regulation, which is vital for hematopoiesis both by providing a physical structure and the non-hematopoietic cellular and extracellular molecules that have very recently been identified as critical components of the HSC niche. OPN is a key molecule in the attraction, retention and regulation of HSC within the endosteal HSC niche (Nilsson *et al.*, 2005).

In the work described in this Chapter, the potential involvement of CD44 in the ATRA and IL-3-induced differentiation pathways was investigated by siRNA knockdown and the expression of CD44 and OPN isoforms in mouse myeloid differentiation models was studied.

4.2 Results

4.2.1 Expression of CD44 and osteopontin in mouse and human myeloid cell lines

To evaluate the potential involvement of CD44 and its ligand OPN in IL-3-driven myeloid differentiation, RT-PCR was used to measure their expression in a variety of mouse cell lines some undergoing differentiation. DNA sequencing of cDNA clones was used to determine which isoforms were expressed. A similar analysis was carried out for the human promyelocytic leukemia line NB4.

4.2.1.1 Expression of CD44 and osteopontin in mouse cell lines undergoing differentiation

Three differentiation models (FDCP-mix multi-potential cells, M1 leukemia cells and WEHI-3B D+ leukemia cells) have been used to study IL-3-induced differentiation in our group by ectopically expressing hIL-3 receptors in these cells (Chen *et al.*, 2009). It was therefore of interest to measure the levels of CD44 and osteopontin in these cells during differentiation. It was found that CD44 mRNA levels were readily detectable in each of these cell lines (Fig.4.1). No major changes in the levels occurred when FDCP-mix and M1 cells expressing hIL-3 receptors underwent differentiation induced by hIL-3. Higher CD44 expression was shown in WEHI-3B D+ cells (the sub-line which differentiates in response to G-CSF) and lower CD44 expression in WEHI-3B D- cells (the non-differentiating sub-line). CD44 expression was high in the Hoxb8-immortalized IL-3-dependent myeloid cell lines B4.1 and 5P4A and also detectable in the IL-3-dependent myeloid line FDCP-1. These cell lines are not capable of IL-3-induced differentiation. Thus CD44 was expressed in the myeloid cell lines capable of cytokine-induced differentiation leaving open the possibility of its involvement in this process. CD44 mRNA expression was not detected in the B cell line CTLL-2 (Fig.4.1). CTLL-2 cells expressing mIL-3Rα SP1/$m\beta_{IL\text{-}3}$ or mIL-3Rα SP2/$m\beta_{IL\text{-}3}$ grown with or without mIL-3 and CTLL-2 expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc grown with or without hIL-3, also did not express CD44 (data not shown). These cells are capable of IL-3-induced growth but not differentiation. This indicates that IL-3 receptor signaling has no effect on CD44 expression in CTLL-2 cells.

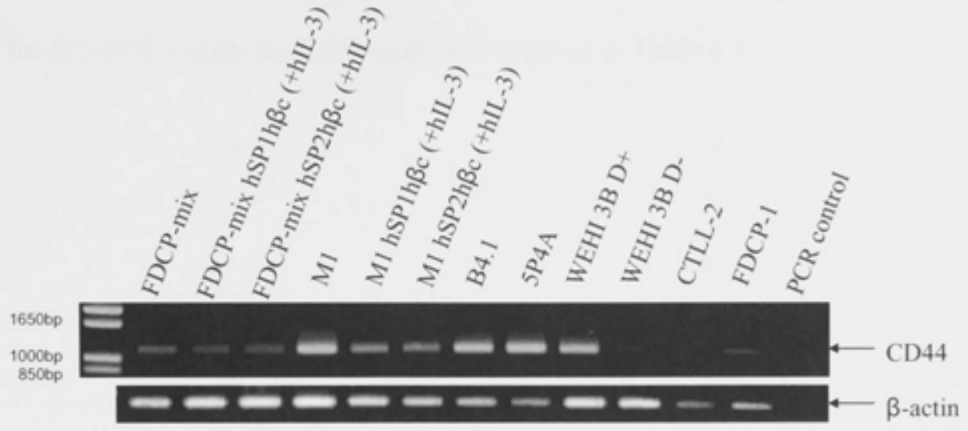


Figure 4.1: The levels of CD44 mRNA in mouse cell lines measured by RT-PCR. FDCP-mix, B4.1, FDCP1 were undergoing IL-3-dependent growth and CTLL-2 was undergoing IL-2 dependent growth. FDCP-mix and M1 cells ectopically expressing hIL-3Rα SP2/hβc were undergoing hIL-3-driven differentiation. RNase-free water was used as PCR control. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

OPN mRNA levels were also readily detectable in the three differentiation models studied (Fig.4.2). The levels did not change significantly when FDCP-mix and M1 cells expressing hIL-3 receptors underwent differentiation induced by hIL-3. Low OPN expression was found in WEHI 3B D+ cells (differentiating sub-line) and high OPN expression in WEHI 3B D- cells (non-differentiating sub-line). The HoxB8 immortalized IL-3-dependent cell lines B4.1 and 5P4A, IL-3-dependent FDCP-1 and IL-2 dependent CTLL-2 cells do not differentiate in the presence of mIL-3. Low OPN expression was detected in B4.1, 5P4A and CTLL-2 cells. No OPN expression was detected in FDCP-1 cells (Fig.4.2).

Thus both CD44 and its ligand OPN were expressed in the myeloid cells undergoing IL-3-induced differentiation making their involvement in the process plausible. A summary of the RT-PCR results for CD44 and OPN is given in Table 4.1.

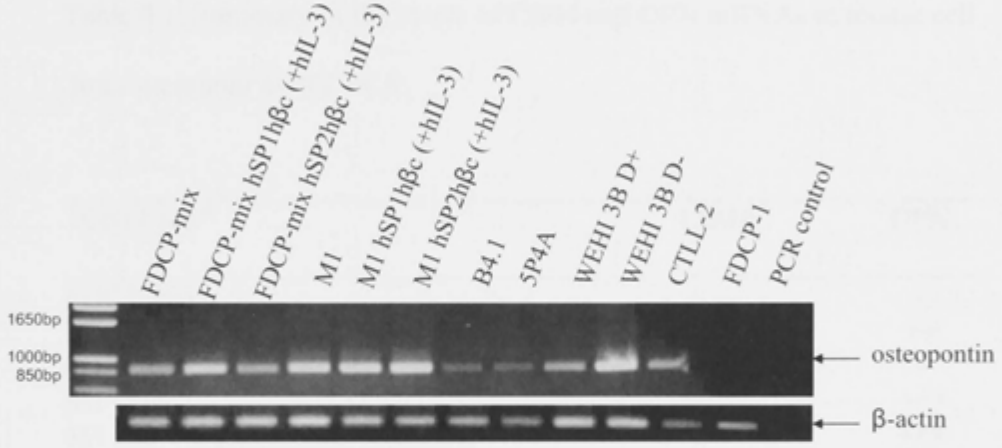


Figure 4.2: The levels of osteopontin mRNA in mouse cell lines measured by RT-PCR. FDCP-mix, B4.1 and FDCP1 cells were undergoing IL-3-dependent growth and CTLL-2 cells were undergoing IL-2 dependent growth. FDCP-mix and M1 cells ectopically expressing hIL-3Rα SP2/hβc were undergoing hIL-3-driven differentiation. RNase-free water was used as PCR control. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

Table 4.1: Summary of the levels of CD44 and OPN mRNAs in mouse cell lines measured by RT-PCR.

Cell lines [a]	CD44	OPN
FDCP-mix	+	+
FDCP-mix hIL-3 Rα SP1 / hβc (+hIL-3)	+	++
FDCP-mix hIL-3 Rα SP2 / hβc (+hIL-3)	+	+
M1	+++	+++
M1 hIL-3 Rα SP1 / hβc (+hIL-3)	+	+++
M1 hIL-3 Rα SP2 / hβc (+hIL-3)	+	+++
WEHI-3B D+	+++	+
WEHI-3B D-	+	+++
B4.1	+++	+/-
5P4A	+++	+/-
CTLL-2	-	+
FDCP-1	+	-

[a] Growth conditions are described in Figs 4.1 and 4.2.

4.2.1.2 Osteopontin expression in a granulocyte-macrophage progenitor undergoing differentiation

SCF ER-Hoxb8 is a conditionally-immortalized granulocyte-macrophage progenitor and was kindly provided by Prof. Mark Kamps. Previous studies have shown that SCF ER-Hoxb8 neutrophil progenitors can grow in the presence of SCF with estrogen and undergo neutrophil differentiation after the removal of estrogen (Wang *et al.*, 2006). Interestingly, no osteopontin expression was detected in the SCF ER-Hoxb8 progenitors grown with estrogen in contrast with the Hoxb8-immortalized B4.1 and 5P4A cells (Fig.4.3). However, the expression of osteopontin mRNA was detected in the differentiation samples of SCF ER-Hoxb8 progenitors (Fig.4.3). Thus in SCF ER-Hoxb8, differentiation correlates with induction of OPN expression.

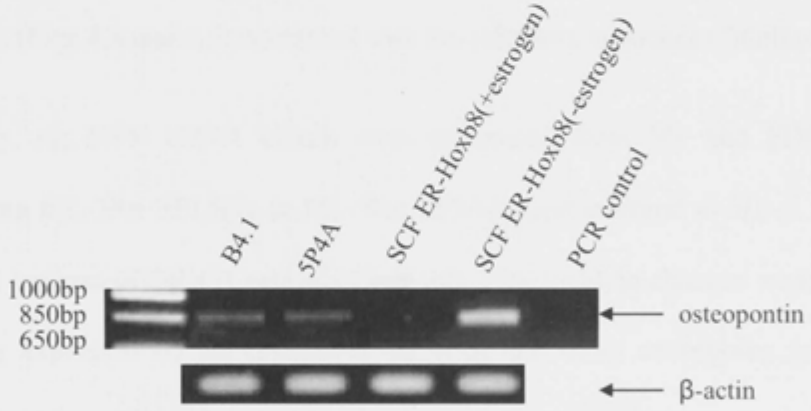


Figure 4.3: Osteopontin mRNA expression in the Hoxb8 immortalized myeloid progenitor SCF ER-Hoxb8 measured by RT-PCR. SCF ER-Hoxb8 was grown in the presence of SCF plus estrogen and differentiated in SCF after the removal of estrogen. For comparison, osteopontin expression was measured in Hoxb8-immortalized B4.1 and 5P4A cells grown with mIL-3. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

4.2.1.3 CD44 and osteopontin isoforms expressed by M1 and FDCP-mix cells undergoing IL-3-supported differentiation

Because there are a number of potential isoforms for CD44 (Ghaffari *et al.,* 1999) and also for OPN (Chae *et al.,* 2009), it was of interest to determine the isoforms expressed and to determine any changes in isoforms between growth and differentiation. Full length sequencing of cDNAs for CD44 and OPN were carried out for M1 and FDCP-mix cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/ hβc and grown in hIL-3.

Six CD44 clones from each cell line were examined, and all were the standard CD44s isoform (Figs.4.4 and 4.5). With each cell line, only growth occurs with hIL-3Rα SP1/ hβc and differentiation occurs with hIL-3Rα SP2/hβc. Thus there were no changes detectable between cells undergoing differentiation and cells undergoing growth. One common amino acid substitution was detected in the cDNA clones from M1 and FDCP-mix cells (Figs.4.4 and 4.5) compared with the reference sequence (Günthert, 1993).

Similarly, six OPN cDNA clones were sequenced from M1 and FDCP-mix cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc and cultured in hIL-3. All were the standard isoform of OPN (Craig and Denhardt, 1991) and no changes were found in the isoforms expressed by the differentiating cells and those undergoing growth. Seven common amino acid substitutions and one amino acid insertion in six clones were detected in M1 cells (Fig.4.6). One common amino acid substitution in six clones was detected in FDCP-mix cells (Fig.4.7) when compared with the reference sequence (Craig and Denhardt, 1991).

```
CD44_M1 hSP2hBc(+hIL-3)  MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50
CD44_M1 hSP1hBc(+hIL-3)  MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50
CD44_M1                  MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50
standard mouse CD44      MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50

CD44_M1 hSP2hBc(+hIL-3)  TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100
CD44_M1 hSP1hBc(+hIL-3)  TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100
CD44_M1                  TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100
standard mouse CD44      TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100

CD44_M1 hSP2hBc(+hIL-3)  CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150
CD44_M1 hSP1hBc(+hIL-3)  CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150
CD44_M1                  CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150
standard mouse CD44      CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150

CD44_M1 hSP2hBc(+hIL-3)  TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPESYILH 200
CD44_M1 hSP1hBc(+hIL-3)  TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPESYILH 200
CD44_M1                  TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPESYILH 200
standard mouse CD44      TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPEGYILH 200

CD44_M1 hSP2hBc(+hIL-3)  TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
CD44_M1 hSP1hBc(+hIL-3)  TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
CD44_M1                  TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
standard mouse CD44      TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
```

Figure 4.4: Alignment of amino acid sequences of mouse CD44 derived from M1 cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc and cultured with hIL-3 and CD44 from M1 parental cells. The full length mouse CD44 cDNA clones were isolated from M1 cells and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

```
CD44_FDCP-mix hSP2hBc(+hIL-3)   MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50
CD44_FDCP-mix hSP1hBc(+hIL-3)   MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50
CD44_FDCP-mix                   MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50
standard mouse CD44             MDKFWWHTAWGLCLLQLSLAHPHQQIDLNVTCRYAGVFHVEKNGRYSISR  50

CD44_FDCP-mix hSP2hBc(+hIL-3)   TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100
CD44_FDCP-mix hSP1hBc(+hIL-3)   TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100
CD44_FDCP-mix                   TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100
standard mouse CD44             TEAADLCQAFNSTLPTMDQMKLALSKGFETCRYGFIEGNVVIPRIHPNAI 100

CD44_FDCP-mix hSP2hBc(+hIL-3)   CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150
CD44_FDCP-mix hSP1hBc(+hIL-3)   CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150
CD44_FDCP-mix                   CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150
standard mouse CD44             CAANHTGVYILVTSNTSHYDTYCFNASAPPEEDCTSVTDLPNSFDGPVTI 150

CD44_FDCP-mix hSP2hBc(+hIL-3)   TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPESYILH 200
CD44_FDCP-mixhSP1hBc(+hIL-3)    TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPESYILH 200
CD44_FDCP-mix                   TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPESYILH 200
standard mouse CD44             TIVNRDGTRYSKKGEYRTHQEDIDASNIIDDDVSSGSTIEKSTPEGYILH 200

CD44_FDCP-mix hSP2hBc(+hIL-3)   TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
CD44_FDCP-mix hSP1hBc(+hIL-3)   TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
CD44_FDCP-mix                   TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
standard mouse CD44             TYLPTEQPTGDQDDSFFIRSTLATRDRDSSKDSRGSSRTVTHGSELAGHS 250
```

Figure 4.5: Alignment of amino acid sequences of mouse CD44 derived from FDCP-mix cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc and cultured with hIL-3 or FDCP-mix parental cells. The full length mouse CD44 cDNA clones were isolated from FDCP-mix cells and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

```
OPN_M1hSP2hBc(+hIL-3) MRLAVICFCLFGIASSLPVKVTDSGSSEEKKLYSLHPDPIATWLVPDPSQ  50
OPN_M1hSP1hBc(+hIL-3) MRLAVICFCLFGIASSLPVKVTDSGSSEEKKLYSLHPDPIATWLVPDPSQ  50
OPN_M1                MRLAVICFCLFGIASSLPVKVTDSGSSEEKKLYSLHPDPIATWLVPDPSQ  50
Standard mouse OPN    MRLAVICFCLFGIASSLPVKVTDSGSSEEK-LYSLHPDPIATWLVPDPSQ  49

OPN_M1hSP2hBc(+hIL-3) KQNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAE 100
OPN_M1hSP1hBc(+hIL-3) KQNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAE 100
OPN_M1                KQNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAE 100
Standard mouse OPN    KQNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAG  99

OPN_M1hSP2hBc(+hIL-3) NEDSVDSDESDESHHSDESDETFTASTQADTFTPIVPTVDVPDGRGDSLA 150
OPN_M1hSP1hBc(+hIL-3) NEDSVDSDESDESHHSDESDETFTASTQADTFTPIVPTVDVPDGRGDSLA 150
OPN_M1                NEDSVDSDESDESHHSDESDETFTASTQADTFTPIVPTVDVPDGRGDSLA 150
Standard mouse OPN    SEDSVDSDESDESHHSDESDETVTASTQADTFTPIVPTVDVPDGRGDSLA 149

OPN_M1hSP2hBc(+hIL-3) YGLRSKSRSFQVSDEQYPDATYEDLTSHMKSGESKESLNVIPVAQLLSMP 200
OPN_M1hSP1hBc(+hIL-3) YGLRSKSRSFQVSDEQYPDATYEDLTSHMKSGESKESLNVIPVAQLLSMP 200
OPN_M1                YGLRSKSRSFQVSDEQYPDATYEDLTSHMKSGESKESLNVIPVAQLLSMP 200
Standard mouse OPN    YGLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQLLSMP 199

OPN_M1hSP2hBc(+hIL-3) SDQDNNGRGSHESSQLDEPSLETHSLEHSKESHESADQSDVIDSQASSKA 250
OPN_M1hSP1hBc(+hIL-3) SDQDNNGRGSHESSQLDEPSLETHSLEHSKESHESADQSDVIDSQASSKA 250
OPN_M1                SDQDNNGRGSHESSQLDEPSLETHSLEHSKESHESADQSDVIDSQASSKA 250
Standard mouse OPN    SDQDNNGKGSHESSQLDEPSLETHRLEHSKESHESADQSDVIDSQASSKA 249
```

Figure 4.6: Alignment of amino acid sequences of mouse OPN derived from M1 cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc and cultured in hIL-3 or M1 parental cells. The full length mouse OPN cDNA clones were isolated from M1 cells and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

```
OPN_FDCP-mix hSP2hBc(+hIL-3)  MRLAVICFCLFGIASSLPVKVTDSGSSEEKLYSLHPDPIATWLVPDPSQK  50
OPN_FDCP-Mix hSP1hBc(+hIL-3)  MRLAVICFCLFGIASSLPVKVTDSGSSEEKLYSLHPDPIATWLVPDPSQK  50
OPN_FDCP-mix                  MRLAVICFCLFGIASSLPVKVTDSGSSEEKLYSLHPDPIATWLVPDPSQK  50
Standard mouse OPN            MRLAVICFCLFGIASSLPVKVTDSGSSEEKLYSLHPDPIATWLVPDPSQK  50

OPN_FDCP-mix hSP2hBc(+hIL-3)  QNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAES 100
OPN_FDCP-Mix hSP1hBc(+hIL-3)  QNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAES 100
OPN_FDCP-mix                  QNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAES 100
Standard mouse OPN            QNLLAPQNAVSSEEKDDFKQETLPSNSNESHDHMDDDDDDDDDDGDHAGS 100

OPN_FDCP-mix hSP2hBc(+hIL-3)  EDSVDSDESDESHHSDESDETVTASTQADTFTPIVPTVDVPNGRGDSLAY 150
OPN_FDCP-Mix hSP1hBc(+hIL-3)  EDSVDSDESDESHHSDESDETVTASTQADTFTPIVPTVDVPNGRGDSLAY 150
OPN_FDCP-mix                  EDSVDSDESDESHHSDESDETVTASTQADTFTPIVPTVDVPNGRGDSLAY 150
Standard mouse OPN            EDSVDSDESDESHHSDESDETVTASTQADTFTPIVPTVDVPNGRGDSLAY 150

OPN_FDCP-mix hSP2hBc(+hIL-3)  GLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQLLSMPS 200
OPN_FDCP-Mix hSP2hBc(+hIL-3)  GLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQLLSMPS 200
OPN_FDCP-mix                  GLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQLLSMPS 200
Standard mouse OPN            GLRSKSRSFQVSDEQYPDATDEDLTSHMKSGESKESLDVIPVAQLLSMPS 200

OPN_FDCP-mix hSP2hBc(+hIL-3)  DQDNNGKGSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSKAS 250
OPN_FDCP-Mix hSP1hBc(+hIL-3)  DQDNNGKGSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSKAS 250
OPN_FDCP-mix                  DQDNNGKGSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSKAS 250
Standard mouse OPN            DQDNNGKGSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSKAS 250
```

Figure 4.7: Alignment of amino acid sequences of mouse OPN derived from FDCP-mix cells expressing hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc and cultured in hIL-3 or FDCP-mix parental cells. The full length mouse OPN cDNA clones were isolated from FDCP-mix cells and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

4.2.1.4 Expression of CD44 and osteopontin in NB4 promyelocytic leukemia cells undergoing ATRA-induced differentiation

CD44 ligation induces differentiation of AML blasts and also of NB4 cells (Charrad *et al.*, 1999; Charrad *et al.*, 2002; Song *et al.*, 2004). It was of interest to determine the levels of expression of CD44 in parental NB4 cells and NB4 cells undergoing ATRA-induced differentiation. When NB4 cells were treated with ATRA, the expression of CD44 mRNA at different times after induction of differentiation was determined by RT-PCR. The expression of CD44 mRNA was readily detectable and did not change significantly before and after induction of differentiation by ATRA (Fig.4.8).

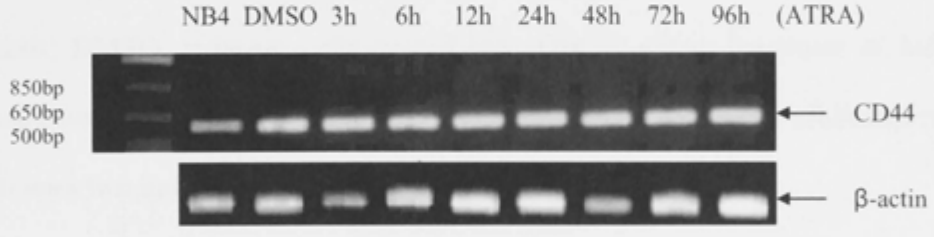


Figure 4.8: The levels of CD44 mRNA in NB4 cells before and after induction of differentiation by ATRA were measured by RT-PCR. ATRA was dissolved in DMSO and included in the medium at a final concentration of 1μM. NB4, indicates untreated cells; DMSO, indicates cells treated with DMSO alone; the times of induction are indicated in the figure. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

Similarly, OPN mRNA expression was measured by RT-PCR in NB4 induced to differentiate by ATRA treatment. OPN mRNA was readily detectable with no significant changes in levels in NB4 cells with or without ATRA treatment (Fig.4.9).

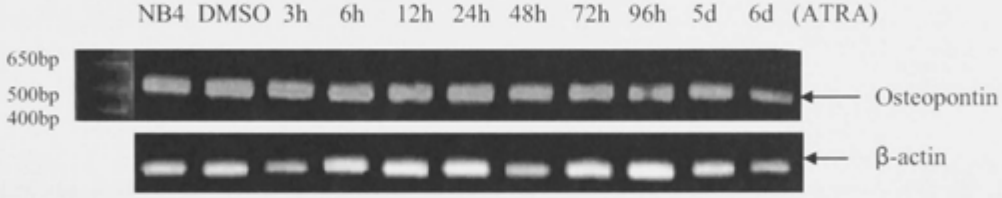


Figure 4.9: The levels of osteopontin mRNA in NB4 cells before and after induction of differentiation by ATRA were measured by RT-PCR. ATRA was dissolved in DMSO and included in the medium at a final concentration of 1μM. NB4, indicates untreated cells; DMSO, indicates cells treated with DMSO alone; the times of induction are indicated in the figure. β-actin was used as an internal standard. Results represent one of at least two independent experiments.

4.2.1.5 Osteopontin isoforms expressed by NB4 cells undergoing ATRA-induced differentiation

DNA sequencing of OPN cDNA clones was carried out. Nine cDNA clones were sequenced from NB4 cells not undergoing differentiation and all were the standard isoform (Kiefer *et al.,* 1989) denoted as OPN_NB4 in Fig.4.10. Single amino acid substitutions (501 T→C, aa V→A) were detected in seven of these clones relative to the reference sequence (Kiefer *et al.,* 1989).

There were changes in OPN isoforms in NB4 cells treated with ATRA. Two cDNA clones out of nine clones sequenced from NB4 cells undergoing differentiation were OPN-isoform C (27 aa deletion; denoted as OPN_NB4+ATRA_1 in Fig.4.10). One clone was OPN-isoform B (14 aa deletion; denoted as OPN_NB4+ATRA_2 in Fig.4.10). Six clones were the standard isoform (Kiefer *et al.,* 1989) and two of these clones had individual amino acid substitutions (815 G→A, aa A→T).

```
OPN_NB4+ATRA_1          MRIAVICFCLLGITCAIPVKQADSGSSEEK-------------------  30
OPN_NB4+ATRA_2          MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDP--  48
OPN_NB4                 MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQ  50
Standard human OPN      MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQ  50

OPN_NB4+ATRA_1          -------QNAVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVDSQ  73
OPN_NB4+ATRA_2          ---------SQKQ---NLLAPQTLPSKSNESHDHMDDMDDEDDDDHVDSQ  86
OPN_NB4                 KQNLLAPQNAVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVDSQ 100
Standard human OPN      KQNLLAPQNAVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVDSQ 100

OPN_NB4+ATRA_1          DSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVV 123
OPN_NB4+ATRA_2          DSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVV 136
OPN_NB4                 DSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVV 150
Standard human OPN      DSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVV 150

OPN_NB4+ATRA_1          PTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITSHMESEELNG 173
OPN_NB4+ATRA_2          PTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITSHMESEELNG 186
OPN_NB4                 PTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITSHMESEELNG 200
Standard human OPN      PTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITSHMESEELNG 200

OPN_NB4+ATRA_1          AYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKA 223
OPN_NB+ATRA_2           AYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKA 236
OPN_NB4                 AYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKA 250
Standard human OPN      AYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKA 250
```

Figure 4.10: Alignment of amino acid sequences of human OPN derived from NB4 cells before and after induction of differentiation by ATRA. The full length human OPN cDNA clones were isolated from NB4 cells and the DNA sequencing was performed using the ABI Big Dye terminator cycle sequencing system with analysis using an ABI 3730 sequencer.

4.2.2 Effect of siRNA knockdown of CD44 on ATRA-induced and IL-3-induced NB4 differentiation

Both AML blasts and NB4 cells can be induced to differentiate by CD44 ligation (Charrad *et al.,* 1999; Charrad *et al.,* 2002; Song *et* al., 2004; Gadhoum *et al.,* 2004). The antibody which is the most effective in inducing differentiation is not commercially available and two requests to the originator of the antibody (Charrad *et al.,* 2002) were unsuccessful so direct experiments on CD44-induced differentiation was not carried out in these experiments. However, siRNA knockdown of CD44 was performed in order to see if CD44 played a critical role in the ATRA-induced or IL-3-induced differentiation of NB4. This cell line was considered to be the most suitable as it had been previously established that differentiation could be induced via CD44 (Charrad *et al.,* 2002).

Real-time PCR showed that siRNA treatment reduced CD44 expression to 92%, 91%, 83% and 77% after 1 to 4 days respectively in NB4 cells (Fig.4.11). FACS analyses showed that the siRNA knockdown of CD44 did not significantly impair the differentiation of NB4 cells induced by ATRA (as evidenced by increases in CD11b expression) (Fig.4.12).

In parallel experiments with NB4 cells ectopically expressing the hIL-3Rα SP1/hβc or hIL-3Rα SP2/hβc, real-time PCR showed that siRNA treatment reduced CD44 expression to 95% and 70% at 1 day and 4 days respectively after siRNA treatment in NB4 cells ectopically expressing hIL-3Rα SP2/hβc and to 92% and 65% at 1 day and 4 days respectively after siRNA treatment in NB4 cells ectopically expressing hIL-3Rα SP1/hβc (Figs.4.13 and 4.15). CD44 knockdown did not impair the partial differentiation induced by hIL-3 as evidenced by increased in CD11b expression (Figs. 4.14 and 4.16).

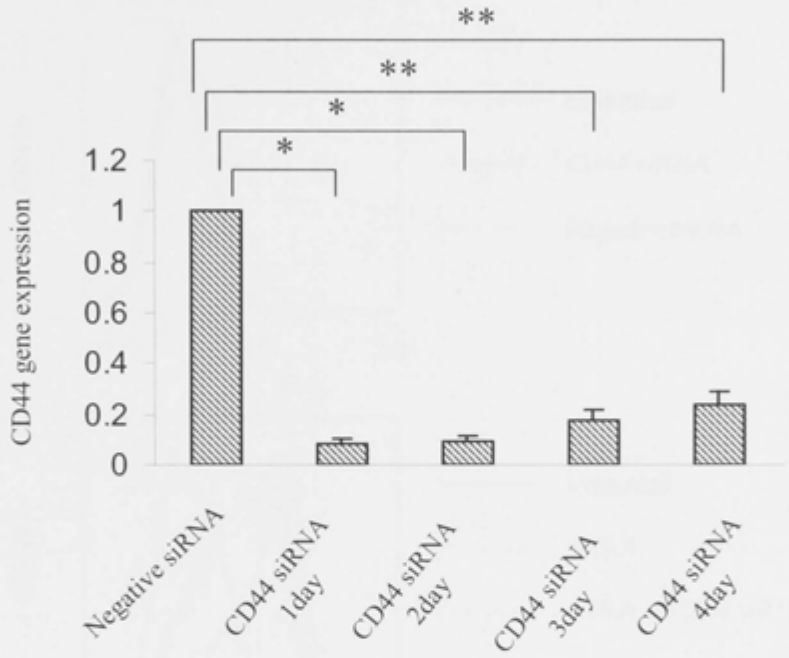


Figure 4.11: The expression of CD44 mRNA in NB4 cells treated with CD44 siRNA and negative siRNA. siRNA knockdown of CD44 mRNA in NB4 cells by CD44 siRNA. NB4 cells were treated with CD44 siRNA and negative siRNA control. The levels of CD44 mRNA in NB4 cells treated with CD44 siRNA for 1 to 4 days was measured by real-time RT-PCR comparison with NB4 cells treated with negative siRNA control. Negative control siRNA (Ambion) do not target any gene product with sequences. Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.001$; **, $p<0.01$ by student's t test.

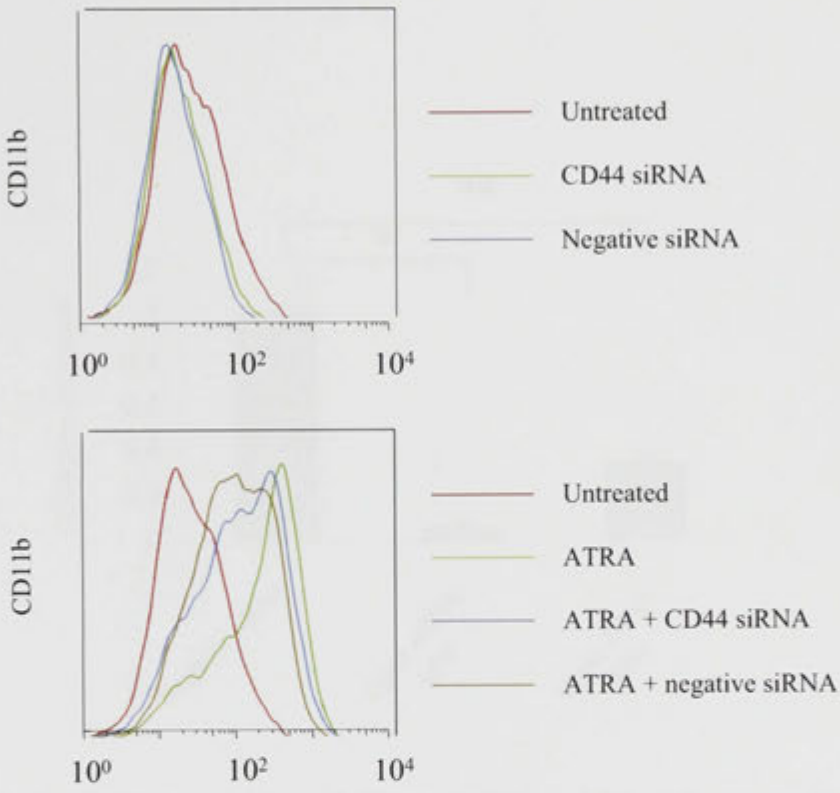


Figure 4.12: CD11b expression in NB4 cells treated with CD44 siRNA and/or ATRA determined by flow cytometry. The effect of CD44 siRNA knockdown on ATRA induced differentiation of NB4 was measured by CD11b expression. NB4 cells were treated with 1 μM ATRA and/or CD44 siRNA or negative siRNA control for 4 days. Results represent one of three independent experiments.

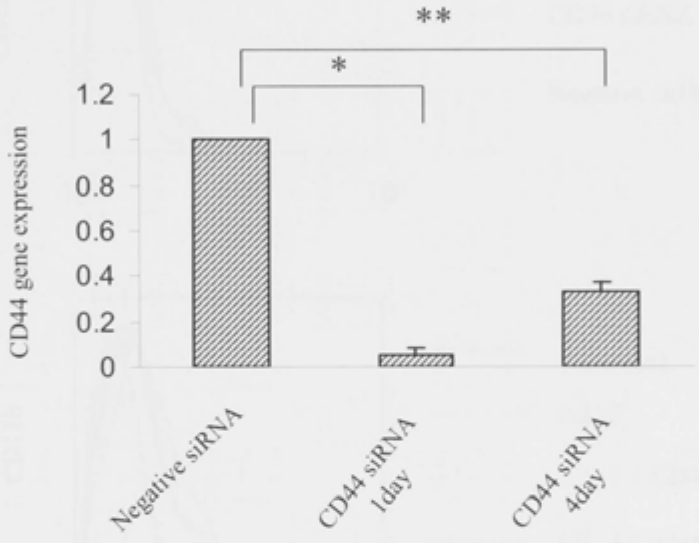


Figure 4.13: siRNA knockdown of CD44 mRNA in NB4 cells expressing hIL-3Rα SP2/hβc by CD44 siRNA. NB4 cells were treated with CD44 siRNA and negative siRNA control. The levels of CD44 mRNA in NB4 cells treated with CD44 siRNA for 1 and 4 days were measured by real-time RT-PCR in comparison with NB4 cells treated with negative siRNA control. Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.001$; **, $p<0.01$ by student's t test.

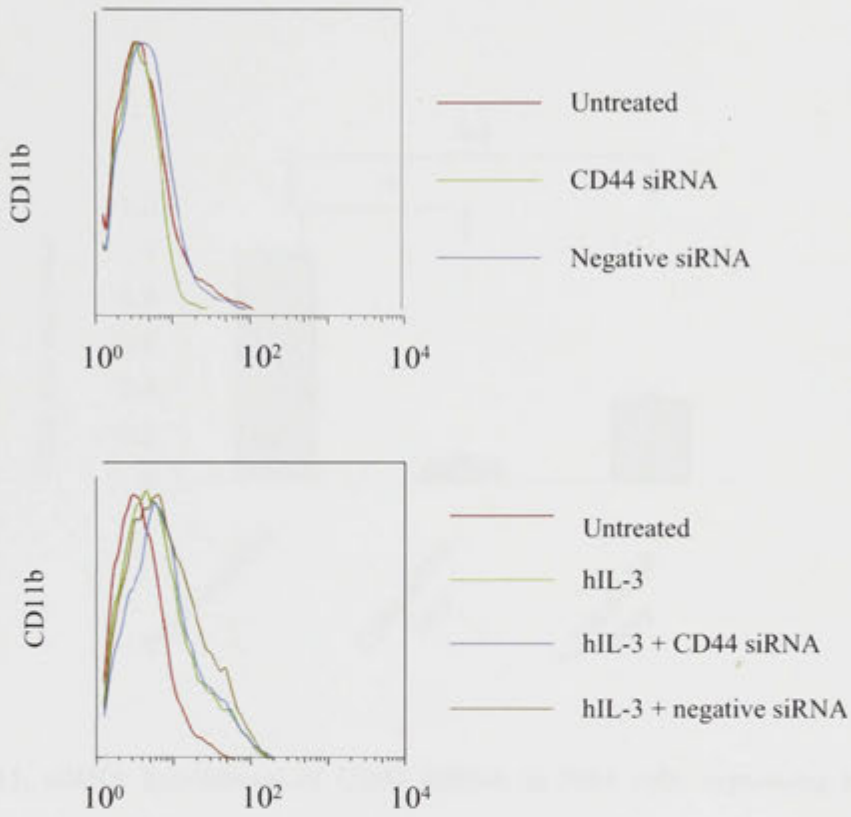


Figure 4.14: The effect of CD44 siRNA knockdown on hIL-3-induced differentiation of NB4 cells ectopically expressing hIL-3Rα SP2/hβc. NB4 cells expressing hIL-3Rα SP2/hβc were treated with hIL-3 and/or CD44 siRNA or negative siRNA control for 4 days. Differentiation was measured by expression of CD11b using flow cytometry. Results represent one of three independent experiments.

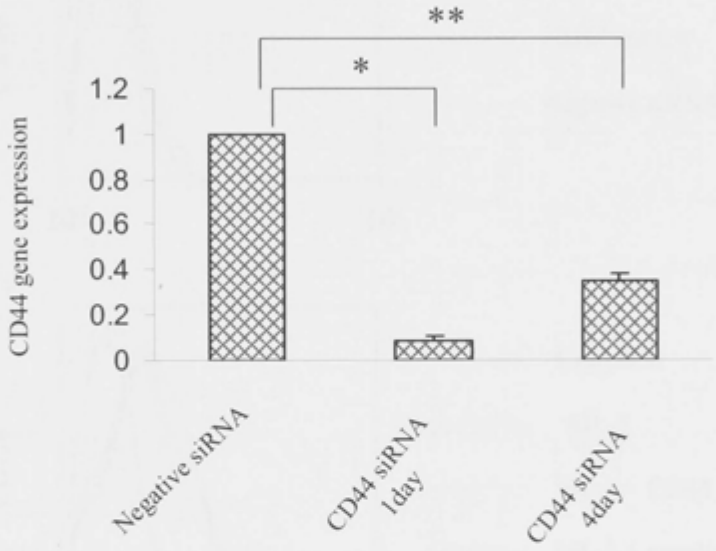


Figure 4.15: siRNA knockdown of CD44 mRNA in NB4 cells expressing hIL-3Rα SP1/hβc by CD44 siRNA. NB4 cells expressing hIL-3Rα SP1/hβc were treated with CD44 siRNA and negative siRNA control. The levels of CD44 mRNA in NB4 cells treated with CD44 siRNA for 1 and 4 days were measured by real-time RT-PCR in comparison with NB4 cells treated with negative siRNA control. Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.001$; **, $p<0.01$ by student's t test.

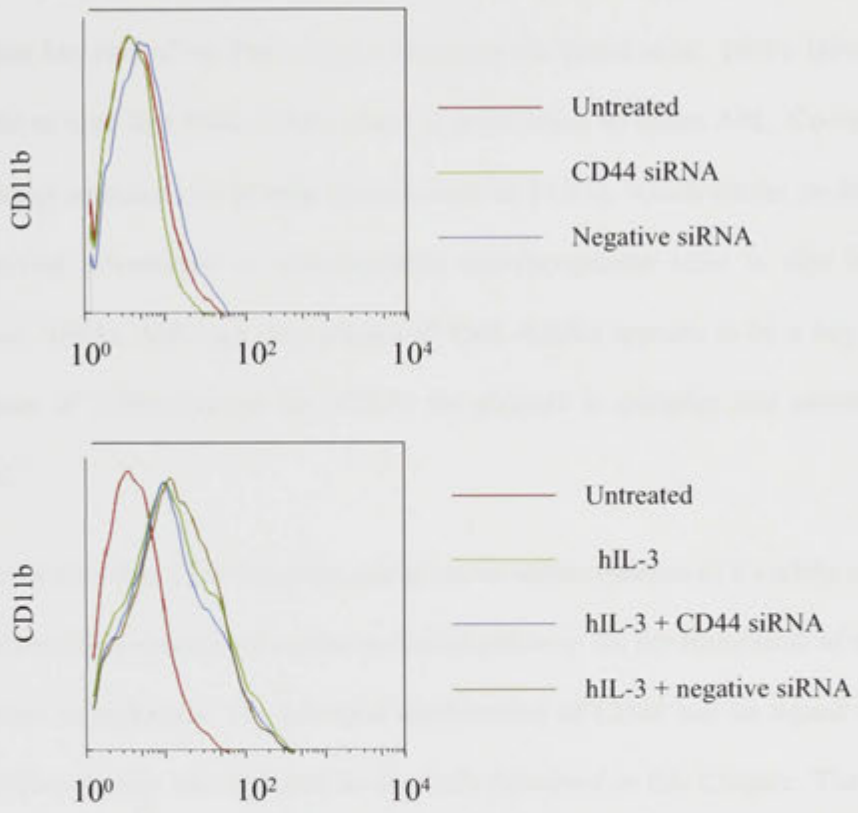


Figure 4.16: The effect of CD44 siRNA knockdown on hIL-3-induced differentiation of NB4 cells ectopically expressing hIL-3Rα SP1/hβc. NB4 cells expressing hIL-3Rα SP1/hβc were treated with hIL-3 and/or CD44 siRNA or negative siRNA control for 4 days. Differentiation was measured by expression of CD11b determined by flow cytometry. Results represent one of three independent experiments.

4.3 Discussion

Differentiation induction with ATRA restores granulocytic differentiation in APL blasts. Much progress has been made in understanding the pathogenesis of APL and in particular the key role of the PML-RARα oncogene (Grignani *et al.,* 1993). However, it is important to note that PML-RARα alone is insufficient to cause APL. Co-operation with activating mutations in protein kinases such as FLT-3, which confer proliferative and/or survival advantages to hematopoietic stem/progenitor cells is also required (Sohal *et al.,* 2003). Although degradation of PML-RARα appears to be a key part of the induction of differentiation by ATRA, the process is complex and incompletely understood.

The demonstration that CD44 ligation can promote differentiation of a variety of AML blasts indicates the existence of another potential pathway for the restoration of myeloid differentiation in leukemia. The potential involvement of CD44 and its ligand OPN in myeloid differentiation was explored in the work described in this Chapter. The CD44-induced differentiation pathway has been shown in AML blasts and NB4 promyelocytic leukemia cells (Charrad *et al.,* 1999; Charrad *et al.,* 2002; Song *et al.,* 2004; Jin *et al.,* 2006) but has not yet been demonstrated in mouse cells. Also, the possible role of the CD44 ligand OPN in this pathway is unclear. In the work described in this Chapter, the expression of CD44 and OPN mRNAs was shown in each of four mouse myeloid cell lines undergoing differentiation so a CD44/OPN differentiation pathway is potentially operative also in mouse cells. The isoforms expressed were the standard isoforms in each case with no changes detectable between growth and differentiation.

SCF ER-Hoxb8 progenitors are conditionally-immortalized granulocyte-macrophage progenitors (GMP), which retain the ability to differentiate into eosinophils, neutrophils

or macrophages. SCF ER-Hoxb8 progenitors can execute neutrophil differentiation in the absence of estrogen (Wang *et al.,* 2006). Interestingly, we found no expression of the CD44 ligand OPN in SCF ER-Hoxb8 progenitors grown with estrogen, while the expression of OPN mRNA was detected in the differentiation samples of SCF ER-Hoxb8 progenitors. OPN has recently been shown to be an important component of early cellular immune responses. The results showed that human monocyte-derived dendritic cells can produce OPN that enhances differentiation, maturation, and survival of DCs by autocrine and/or paracrine pathways (Renkl *et al.,* 2005). The involvement of CD44 in SCF ER-Hoxb8 differentiation has not previously been studied. Further experiments are required to demonstrate the functional significance of the OPN induction demonstrated in the present work.

CD44 ligation induces differentiation of AML blasts and also of NB4 cells (Charrad *et al.,* 1999; Charrad *et al.,* 2002; Song *et al.,* 2004; Jin *et al.,* 2006). In the work described in this Chapter, the expression of CD44 and OPN in parental NB4 cells and NB4 cells undergoing ATRA-induced differentiation was measured. The expression of CD44 and OPN mRNAs were readily detectable and the levels of expression did not change significantly before and after induction of differentiation by ATRA.

Previous studies have shown that CD44 is a receptor for OPN and that OPN is a glycoprotein associated with many physiological and pathological processes including cell adhesion, apoptosis, and tumour metastasis (Haylock and Nilsson, 2006). Human OPN gene is subject to alternative splicing, which yields three distinct isoforms. OPN-A constitutes the full-length transcript, whereas OPN-B and OPN-C result from alternative splicing (Young *et al.,* 1990; Saitoh *et al.,* 1995; He *et al.,* 2006). The relative expression of the three isoforms in human tumors and their function is not well characterized. While one study suggested that OPN-C was selectively expressed in

invasive breast cancer and more likely to support anchorage independent tumor growth than OPN-A (Mirza *et al.,* 2008), another study indicated that OPN-C was less likely to promote cell migration and invasion than the other two OPN isoforms in mesothelioma cells (Ivanov *et al.,* 2009). The role of OPN and its splice variants in acute leukemia is not clear.

In the present work, DNA sequencing of OPN cDNA clones from NB4 cells revealed a change in OPN isoform expression after treatment with ATRA. While parental NB4 cells solely expressed the full length OPN-A isoform, differentiating NB4 cells additionally expressed the OPN-C and OPN-B isoforms. The significance of this finding is not clear, and requires further experiments to evaluate.

CD44 ligation is a potent stimulus for differentiation of promyelocytic leukemia blasts (Charrad *et al.,* 1999) and reduces the levels of the PML-RARα protein more effectively than ATRA. This raises the question as to whether CD44 might be involved in ATRA-induced differentiation. siRNA knockdown of CD44 in NB4 cells did not affect ATRA-driven or IL-3-driven differentiation suggesting that CD44 is not a critical component of either of these differentiation pathways. Thus together with the work described in Chapter 3, these results suggest that the mechanisms involved in the induction of differentiation via the ATRA-driven, IL-3-driven and CD44-promoted differentiation pathways are independent.

Chapter 5

IL-3-induced differentiation of the granulocyte-macrophage progenitor SCF ER-Hoxb8

5.1 Introduction

IL-3, GM-CSF and IL-5 are major hematopoietic cytokines and play an important role in hematopoiesis associated with allergic inflammation or immune responses (Arai *et al.,* 1990). The mechanisms regulating cytokine-driven myeloid differentiation are poorly understood and there is a need for the development of new models of cytokine-supported differentiation which can be subjected to detailed biochemical analysis.

The work described in this Chapter is concerned with the mechanisms of IL-3-driven myeloid differentiation. IL-3 promotes both self-renewal and differentiation of early multipotential progenitors and is involved in inducible hematopoiesis in response to infections. In humans, IL-3 utilizes the βc receptor which is shared with IL-5 and GM-CSF. In mice, there is a shared βc (mβc) receptor but also an IL-3-specific β receptor ($\beta_{IL\text{-}3}$) (Itoh *et al.,* 1990). Recently, our group identified of a new isoform of IL-3Rα (designated SP2), present in mouse and human hematopoietic cells, which lacks domain 1 of the full-length receptor (SP1) (Chen *et al.,* 2009). The ability of the IL-3Rα SP2 and SP1 to promote differentiation of the multi-potential hematopoietic cell line FDCP-mix and the mouse myeloid leukemia line M1 was previously investigated. Of great interest is the finding that the human IL-3Rα SP2 together with hβc promotes the differentiation of multi-potential FDCP-mix cells into basophil-like cells in contrast to the IL-3Rα SP1 isoform which promotes self-renewal. Human IL-3Rα SP2 also directs the differentiation of the myeloblastic M1 leukemia line into monocytes whereas IL-3Rα SP1 does not (Chen *et al.,* 2009). How IL-3 achieves its dual signaling role of promoting self-renewal and differentiation has remained a mystery. The identification of a novel isoform of the IL-3 receptor with activity in differentiation gives a new insight into these processes.

Although the ectopic expression of the human IL-3 receptor in mouse cells enabled our group to study the role of the human IL-3Rα isoforms in promoting differentiation, it is highly desirable to develop a model for studying the role of the mIL-3R in driving myeloid differentiation. This is because mice are the key experimental system for studying hematopoiesis and also for studying allergic inflammation in asthma models. Thus *in vitro* findings can potentially be tested further *in vivo*.

The evaluation of three potential models for studying the mechanisms of mIL-3R-driven differentiation is described in the present Chapter. Initially ectopic expression of mIL-3R subunits in the M1 leukemia line was tested as it has very low levels of endogenous mIL-3 receptors. Also, ectopic expression of mIL-3R subunits was tested in human NB4 cells. Neither cells yielded satisfactory models. Subsequently SCF ER-Hoxb8 cells were shown to be an excellent conditional model for studying the mechanisms of IL-3-driven differentiation of mouse granulocyte macrophage progenitors.

Hox oncoproteins can arrest myeloid differentiation and enforce self-renewal of factor-dependent myeloid progenitors (Knoepfler *et al.,* 2001). The estrogen-regulated Hoxb8 (ER-Hoxb8) has the estrogen-binding domain of the estrogen receptor (ER) fused to the N-terminus of Hoxb8 so that the activity of Hoxb8 is regulated by estrogen levels in the medium (Wang *et al.,* 2006). ER-Hoxb8 can immortalize neutrophil or macrophage progenitors which execute normal differentiation in the absence of estrogen and grow without differentiation in the presence of estrogen. Wang *et al.,* (2006) reported the isolation of the conditionally immortalized granulocyte-macrophage progenitor line SCF ER-Hoxb8. This cell line grows continuously in the presence of SCF and estrogen but differentiates exclusively to neutrophils in the absence of estrogen. When grown in the presence of SCF plus GM-CSF, without estrogen, macrophages as well as neutrophils are formed (Wang *et al.,* 2006). Whether this cell line responds to IL-3 was

not previously reported. As described below, mIL-3 alone was shown to support the growth and differentiation of SCF ER-Hoxb8 cells making this cell line a potentially valuable model for studying mIL-3-driven differentiation. Following the successful validation of the SCF ER-Hoxb8 model, it was used in preliminary experiments aimed at determining the mechanisms involved in IL-3-driven differentiation. These studies included determination of the IL-3Rα isoforms utilized by SCF ER-Hoxb8, measurement of the IL-3 signaling pathways during self-renewal and differentiation and determination of the transcription factors and miRNAs involved in the switch to differentiation.

Transcription factors are known to play a major role in regulating hematopoietic differentiation and lineage commitment (Friedman, 1996). Among the master transcription factors for myelopoiesis are C/EBPα, PU.1, GFI1, IRFs, SCL/TAL1, and RUNX1/AML1. In human AML many of these transcription factors are involved in chromosomal translocations or contain somatic mutations (Tenen, 2003; Rosenbauer and Tenen, 2007). The transcription factors control lineage determination during differentiation in conjunction with miRNAs (microRNAs). miRNAs, a class of ~22 nucleotide non-coding RNAs, were discovered to play important regulatory roles in mammalian hematopoiesis (Chen and Lodish, 2005). miRNAs regulate the translation and stability of specific mRNAs and are believed to significantly contribute to the regulation of lineage commitment and differentiation. They provide a level of control beyond the transcription factors and can potentially control the levels of mRNAs of hundreds of genes (Fatica *et al.,* 2008). Recently, the up-regulation of miR-223 has been reported to be associated with up-regulation of PU.1 and C/EBPα in steady-state blood cell formation (Fukao *et al.,* 2007). It is believed that C/EBPα has a specific function in granulopoiesis: replacing nuclear factor I-A on the miR-223 promoter and up-regulating

miR-223 transcription. The increase in miR-223 levels results in down-regulation of the nuclear factor I-A protein and stimulation of granulocytic differentiation (Fatica *et al.*, 2006). Thus, it is of great interest to see if the differentiation promoted by IL-3R signaling, which may normally be utilized for inducible hematopoiesis in response to infections, may employ similar mechanisms to those involved in normal blood cell formation. The conditional differentiation of SCF ER-Hoxb8 allowed the investigation of the critical transcription factors and miRNAs induced during the onset of differentiation of ER-Hoxb8 progenitors.

5.2 Results

5.2.1 Differentiation of M1 cells ectopically expressing mIL-3Rs

M1 is a mouse myeloblastic leukemia line which can undergo cytokine-induced differentiation (Smith *et al.*, 1997). Previous studies have indicated that M1 cells express low levels of mIL-3Rα SP1 and very low levels of $m\beta_{IL\text{-}3}$/ mβc (Chen *et al.*, 2009) suggesting the possibility that the function of the mIL-3R in differentiation could be investigated by ectopic expression. mIL-3 does not induce M1 to differentiate. Ectopic expression of hIL-3Rα SP2/hβc allows hIL-3-driven differentiation of the M1 cells into monocytes whereas expression of hIL-3Rα SP1/hβc does not (Chen *et al.*, 2009). Human GM-CSFRα/hβc expression also gives differentiation of M1 cells in the presence of hGM-CSF (Smith *et al.*, 1997).

In the present work, Western blotting together with deglycosylation of samples was used to investigate expression of the mIL-3Rα isoforms in M1 cells (Fig.5.1). A low level of mIL-3Rα SP1 was detected but no mIL-3Rα SP2. The presence of low endogenous levels of mIL-3Rα SP1 required care with controls, but it was considered

worthwhile to proceed with ectopic expression of mIL-3R subunits to evaluate the potential of M1 for studying mIL-3-driven myeloid differentiation.

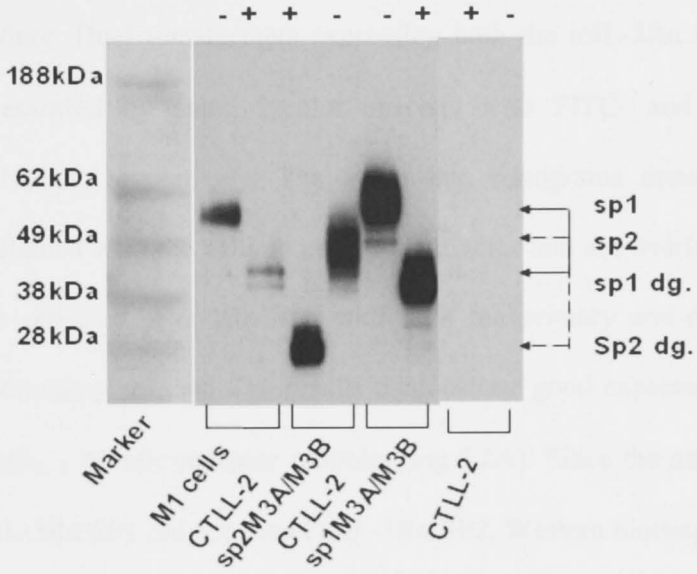


Figure 5.1: Western blot analysis of the endogenous mIL-3Rα SP2 and SP1 protein expression in mouse M1 cells. CTLL-2 cells transfected with mSP2 or SP1 were used as positive controls. Proteins samples were analyzed with (+) or without (-) deglycosylation (*dg*) treatment using peptide *N*-glycosidase F (*PNGase F*). Results represent one of at least two independent experiments.

5.2.1.1 Ectopic expression of mIL-3Rα and β subunits in M1 cells

mIL-3R subunits were ectopically expressed in M1 cells by stable transfection and mIL-3Rα and β subunit expression was verified using FACS and Western blotting (Fig.5.2). Ectopic receptor expression was measured by flow cytometry using a 2-step antibody labelling procedure. Dual transfectants expressing both the mIL-3Rα together with β subunit were assessed by using 2-color analysis with FITC- and PE-conjugated secondary antibodies, respectively. The green line histograms denote non-specific fluorescence obtained with the isotype control antibodies and are overlaid with the red line histograms obtained after labelling with both the primary anti-receptor subunit antibody and secondary reagent. The results demonstrate good expression of the mIL-3Rα SP1 and $m\beta_{IL\text{-}3}$ or mβc receptor subunits (Fig.5.2A). Since the antibody used for detection of mIL-3Rα SP1 did not detect mIL-3Rα SP2, Western blotting was employed using a polyclonal antibody which detected both isoforms. The results showed high expression of both the mIL-3Rα SP1 and SP2 isoforms (Fig.5.2B).

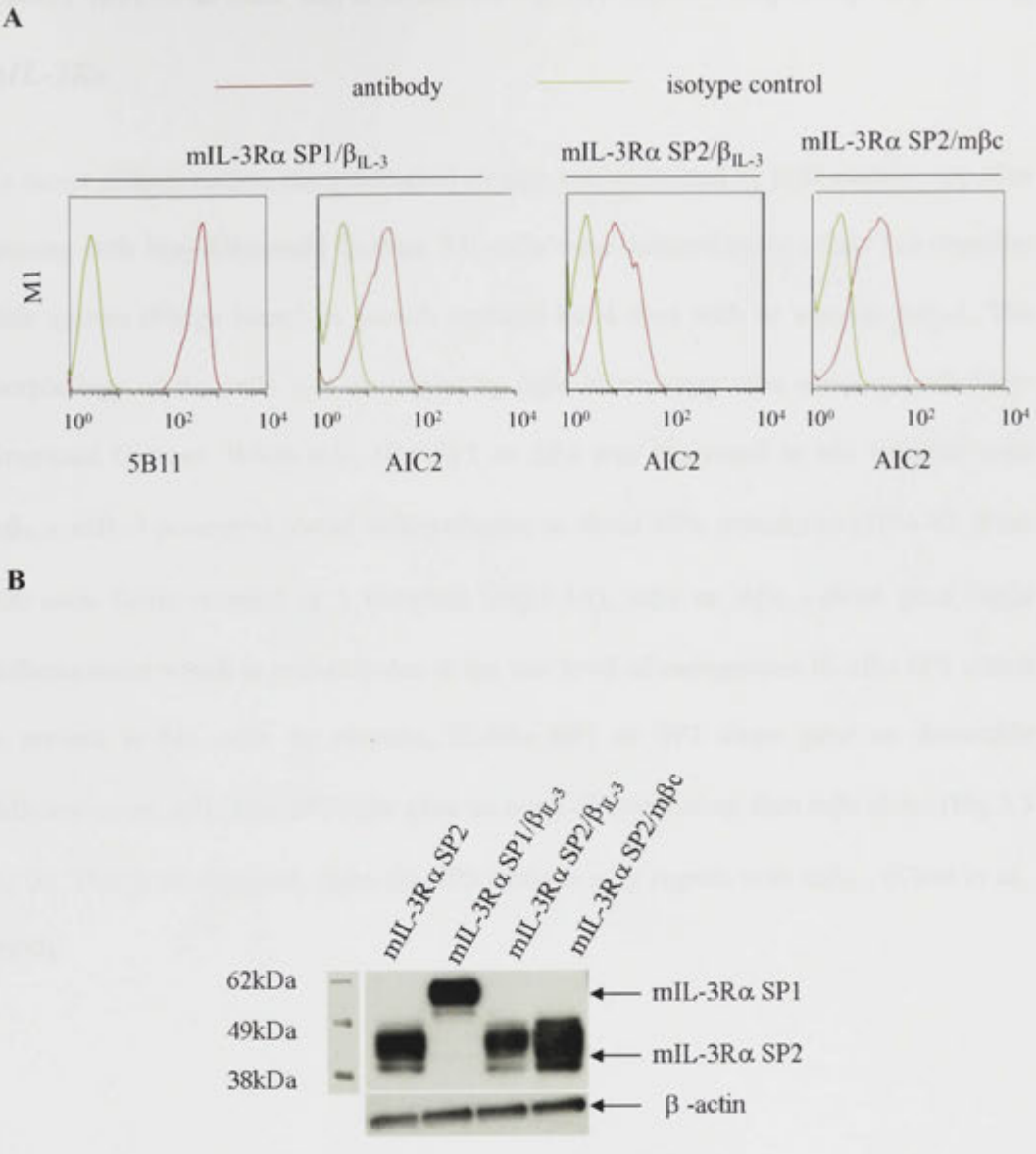


Figure 5.2: Detection of the expression of mIL-3Rα SP1 or mIL-3Rα SP2 and mouse β_{IL-3} or βc in M1 cells by FACS or Western blotting. **A:** Detection of mIL-3Rα SP1 or mouse β_{IL-3} or βc in M1 cells by FACS. AIC2, rat anti-mouse β_{IL-3} /βc monoclonal antibody; 5B11, rat anti-mouse IL-3Rα monoclonal antibody. **B:** Detection of mIL-3Rα SP2 expression by Western blot analysis. Mouse β-actin was used as internal protein loading control. Results represent one of at least two independent experiments.

5.2.1.2 mIL-3-driven differentiation of M1 cells ectopically expressing mIL-3Rs

To detect differentiation, morphological change was examined by light microscopy after staining with May-Grunwald Giemsa. M1 cells were cultured using a Lab-Tek chamber slide system (Nalge Nunc) in growth medium for 4 days with or without mIL-3. The morphology of the cells was examined by light microscopy after staining with May-Grunwald Giemsa. When mIL-3Rα SP1 or SP2 was expressed in M1 together with $m\beta_{IL\text{-}3}$, mIL-3 promoted partial differentiation to about 10% monocytes (10% ±2, from 100 cells fields counted in 3 samples) (Fig.5.3A). mβc or $m\beta_{IL\text{-}3}$ alone gave slight differentiation which is probably due to the low level of endogenous IL-3Rα SP1 which is present in M1 cells. In contrast, IL-3Rα SP1 or SP2 alone gave no detectable differentiation. mIL-3Rα SP2/mβc gave no more differentiation than mβc alone (Fig.5.3 A, B). This is as expected, since the SP2 isoform only signals with $m\beta_{IL\text{-}3}$ (Chen *et al.*, 2009).

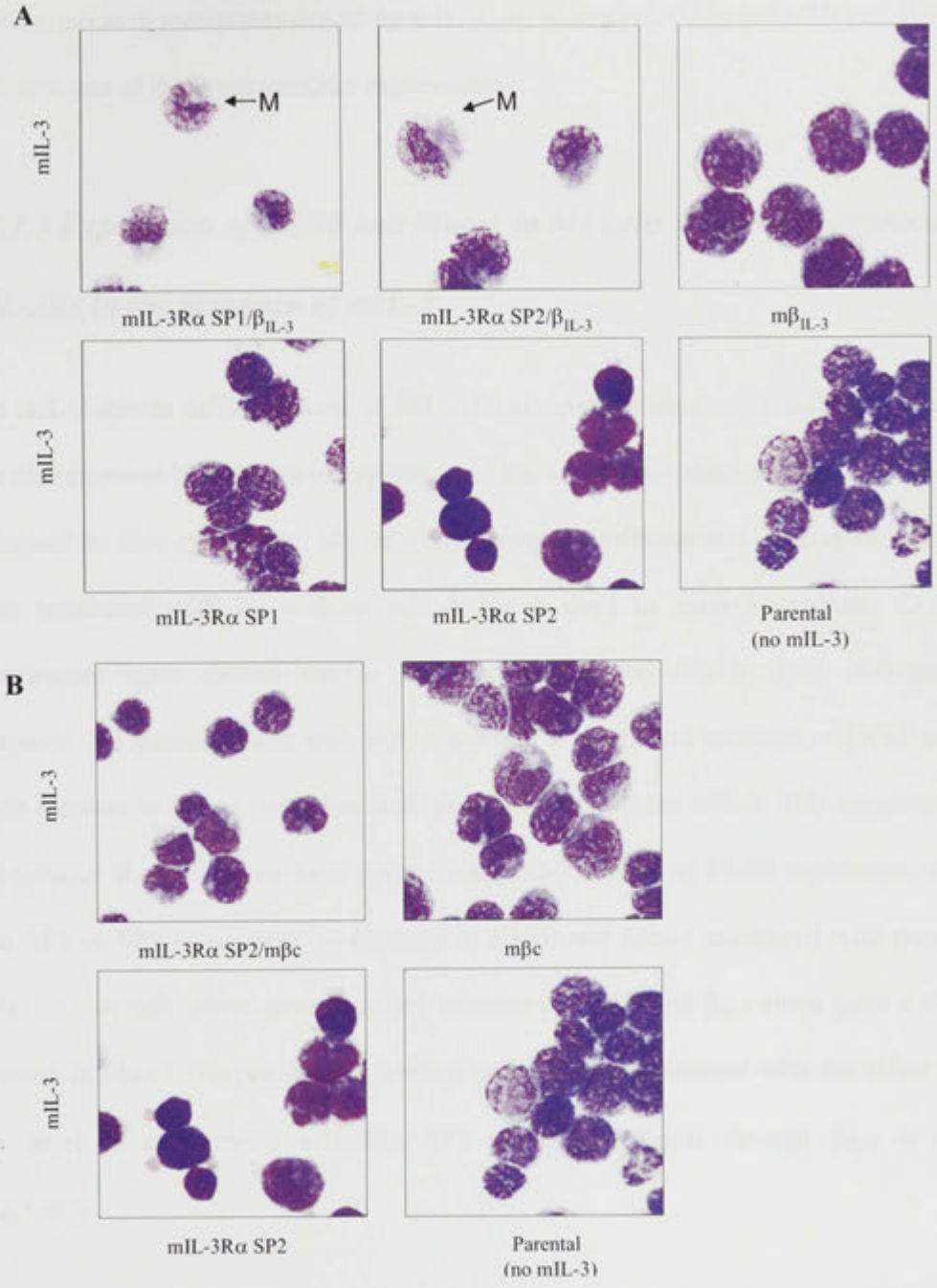


Figure 5.3: Morphological changes in M1 cells expressing different mIL-3R subunits after 4 days culture with mIL-3. Responses to mIL-3 of M1 cells expressing the mIL-3Rα SP1 or SP2 isoforms with and without mβ$_{IL-3}$ or mβc after 4 days incubation were compared with responses to M1 parental cells without mIL-3. Morphological examination was made by light microscopy after May-Grunwald-Giemsa staining. M1

cells ectopically expressing mIL-3Rα SP1/ $m\beta_{IL-3}$ or SP2/ $m\beta_{IL-3}$ gave partial differentiation to monocytes driven by mIL-3. *M,* monocyte. Scale bar = 10 μm. Results represent one of three independent experiments.

5.2.1.3 Expression of F4/80 and Mac-1 in M1 cells ectopically expressing mIL-3Rs in the presence of mIL-3

The mIL-3-driven differentiation of M1 cells ectopically expressing mIL-3R subunits was also assessed by changes in expression of the cell surface markers F4/80 and Mac-1 measured by flow cytometry. M1 cells expressing the different mIL-3 receptor subunits were incubated with or without mIL-3 for 4 days in growth medium. Control experiments were carried out to measure background effects from endogenous receptors. M1 parental cells with mIL-3 showed a significant increase in F4/80 and a slight increase in Mac-1 compared with parental cells without mIL-3. This suggests that endogenous IL-3 receptors have some background effects on F4/80 expression. mIL-3Rα SP1 or SP2 alone gave no increase in F4/80 and Mac-1 compared with parental cells. β_{IL-3} or mβc alone gave a slight increase in F4/80 and β_{IL-3} alone gave a slight increase in Mac-1 compared with parental cells. This is consistent with the effect of a low level of endogenous mIL-3Rα SP1 which can signal through β_{IL-3} or mβc (Fig.5.4C).

Differentiation was shown by increased expression of the surface markers F4/80 and Mac-1. Expression of mIL-3Rα SP1/β_{IL-3} gave an obvious increase in F4/80 and Mac-1 expression and expression of mIL-3Rα SP2/β_{IL-3} gave an obvious increase in F4/80 and a slight increase in Mac-1 expression compared with parental cells (Fig.5.4A). This is in good agreement with the observed morphological changes (Fig.5.3A). Expression of mIL-3Rα SP2/mβc gave a slight increase in F4/80 and Mac-1 expression compared with

parental cells which was quite similar to expression of mβc alone, consistent with the previous finding (Chen *et al.*, 2009) that mIL-3Rα SP2 does not signal through mβc (Fig.5.4B).

Although there were clear effects of the mIL-3R on differentiation, the differentiation was incomplete and the endogenous levels of IL-3Rα SP1 gave background effects. Thus M1 was not considered satisfactory as a mIL-3 differentiation model.

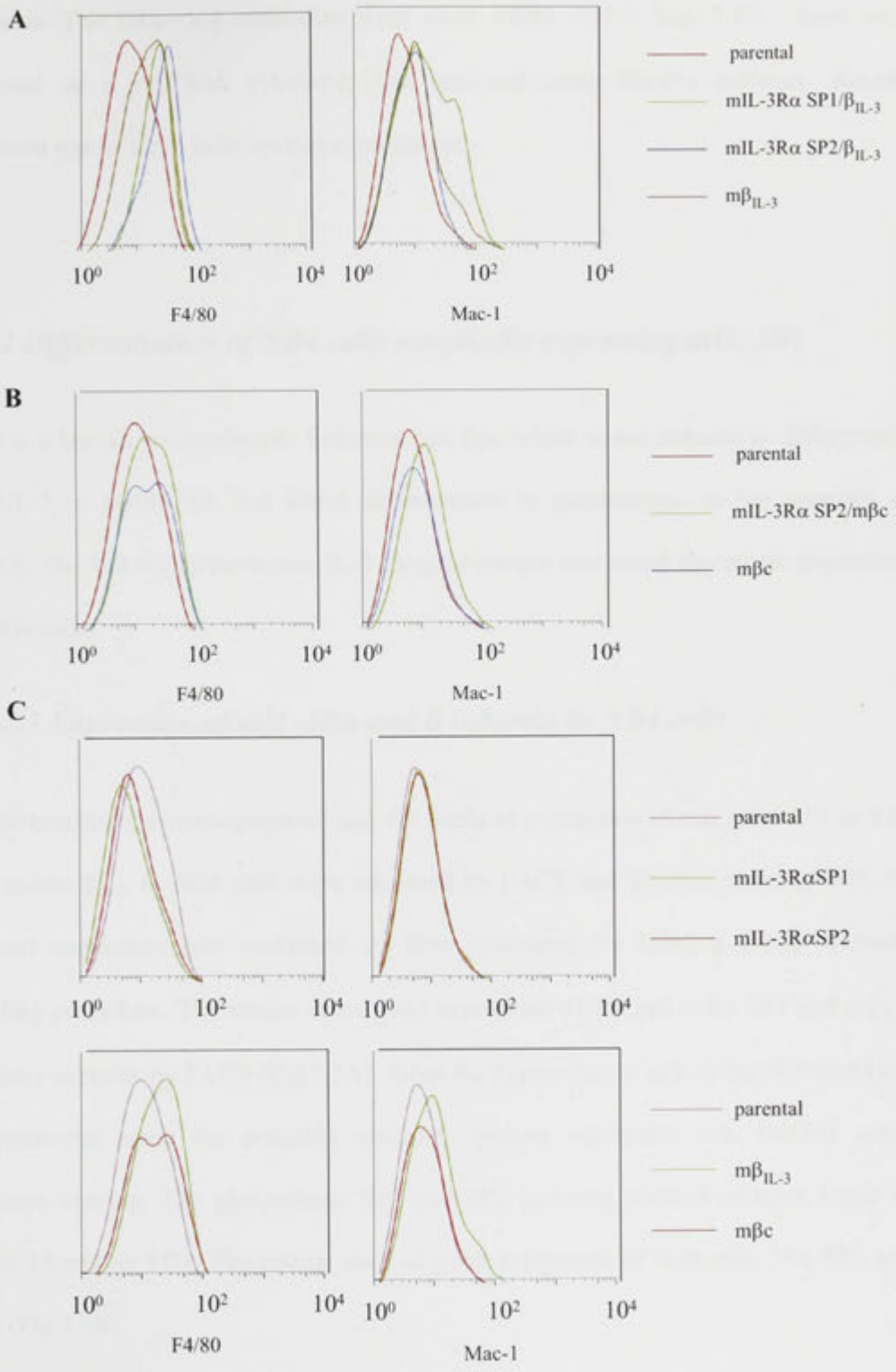


Figure 5.4: FACS analysis of expression of the surface markers F4/80 and Mac-1. M1 cells expressing the different mIL-3R subunits were cultured with mIL-3 for 4 days.

Expression of Mac-1 and F4/80 in M1 cells were measured by FACS as described in Methods. The following antibodies were used: F4/80- FITC, Mac-1-PE. Data were acquired on a FACSort cytometer and analysed using FlowJo software. Results represent one of three independent experiments.

5.2.2 Differentiation of NB4 cells ectopically expressing mIL-3Rs

NB4 is a human promyelocytic leukemia cell line which is not induced to differentiate by hIL-3 or hGM-CSF, but which differentiates to granulocytes in the presence of ATRA. The activity of the mouse IL-3 receptor system was tested by ectopic expression in NB4 cells.

5.2.2.1 Expression of mIL-3Rα and β subunits in NB4 cells

Stable transfectants were prepared and the levels of expression of mIL-3Rα SP1 or SP2 and mouse $\beta_{IL\text{-}3}$ in NB4 cells were measured by FACS and Western blotting. mIL-3R subunit expression was confirmed by flow cytometry by using a 2-step antibody labeling procedure. The results show good expression of the mIL-3Rα SP1 and $m\beta_{IL\text{-}3}$ receptor subunits by FACS (Fig.5.5A). Since the expression of mIL-3 Rα SP2 could not be measured using the available antibody, protein expression was verified using Western blotting. The glycosylated SP1 and SP2 isoforms showed receptor bands of about 54 and 41 kDa. The results showed good expression of both mIL-3Rα SP1 and SP2 (Fig.5.5B).

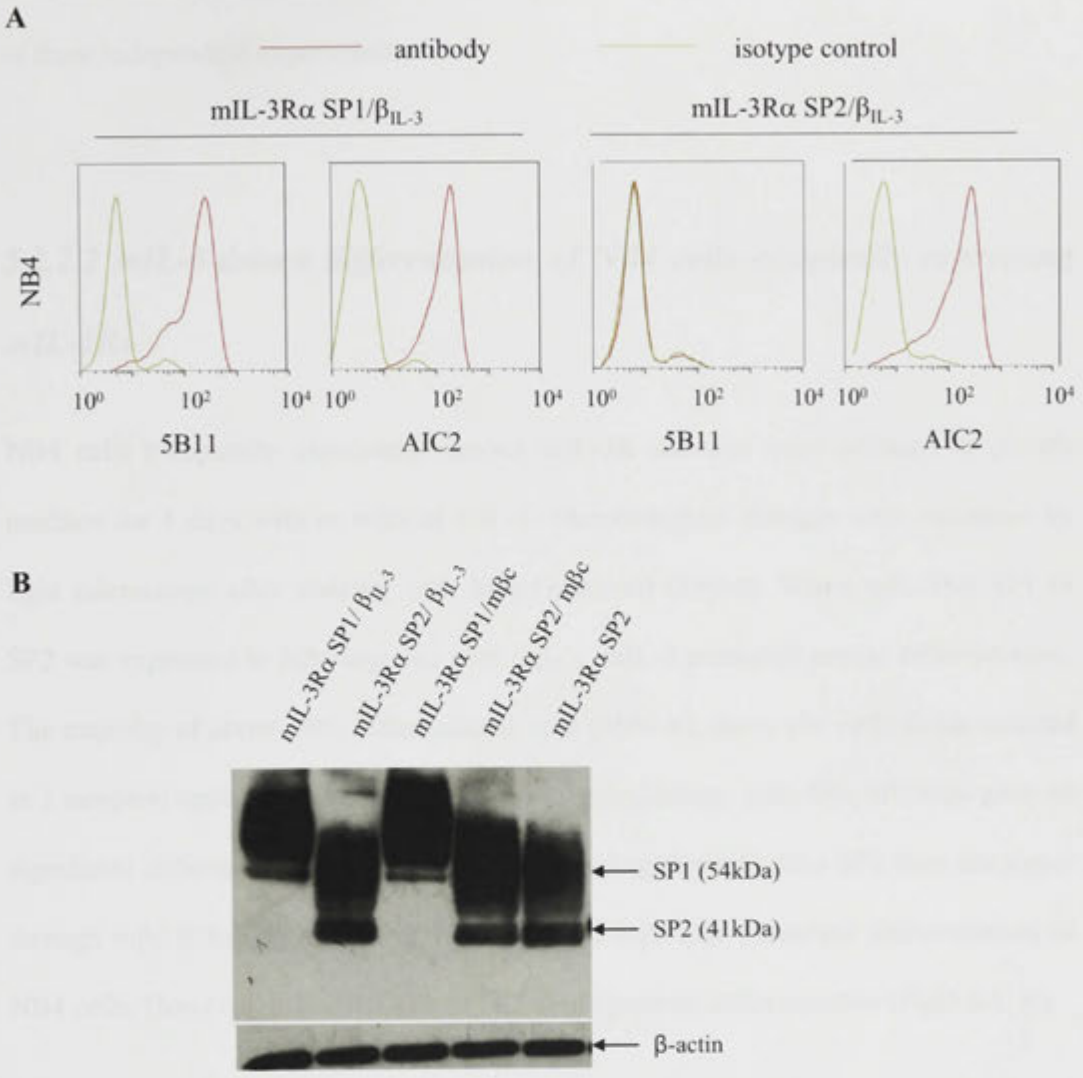


Figure 5.5: Detection of the expression of mIL-3Rα SP1 or mIL-3Rα SP2 and mouse β_{IL-3} in NB4 cells by FACS or Western blotting. **A:** Detection of mIL-3Rα SP1 or mouse β_{IL-3} in NB4 cells by FACS. AIC2, rat anti-mouse β_{IL-3} /βc monoclonal antibody; 5B11, rat anti-mouse IL-3Rα monoclonal antibody. Dual transfectants expressing both the mIL-3 Rα together with β subunit were assessed by using 2-color analysis with FITC- and PE-conjugated secondary antibodies, respectively. The green line histograms denote nonspecific fluorescence obtained with the isotype control antibodies and are overlaid with the red line histograms obtained after labeling with both the primary anti receptor subunit

antibody and secondary reagent. **B:** Detection of mIL-3Rα SP2 expression by Western blot analysis. Mouse β-actin was used as internal protein loading control. Results represent one of three independent experiments.

5.2.2.2 mIL-3-driven differentiation of NB4 cells ectopically expressing mIL-3Rs

NB4 cells ectopically expressing various mIL-3R subunits were cultured in growth medium for 4 days with or without mIL-3. Morphological changes were examined by light microscopy after staining with May-Grunwald Giemsa. When mIL-3Rα SP1 or SP2 was expressed in NB4 together with $m\beta_{IL\text{-}3}$, mIL-3 promoted partial differentiation. The majority of about 10% differentiated cells (10% ±2, from 100 cells fields counted in 3 samples) appeared to be monocyte-like by morphology. mIL-3Rα SP2/mβc gave no significant differentiation, consistent with the finding that mIL-3Rα SP2 does not signal through mβc (Chen *et al.*, 2009). mIL-3Rα SP1/mβc also promoted differentiation of NB4 cells. However, mIL-3Rα SP1 or SP2 alone gave no differentiation (Fig.5.6A, B).

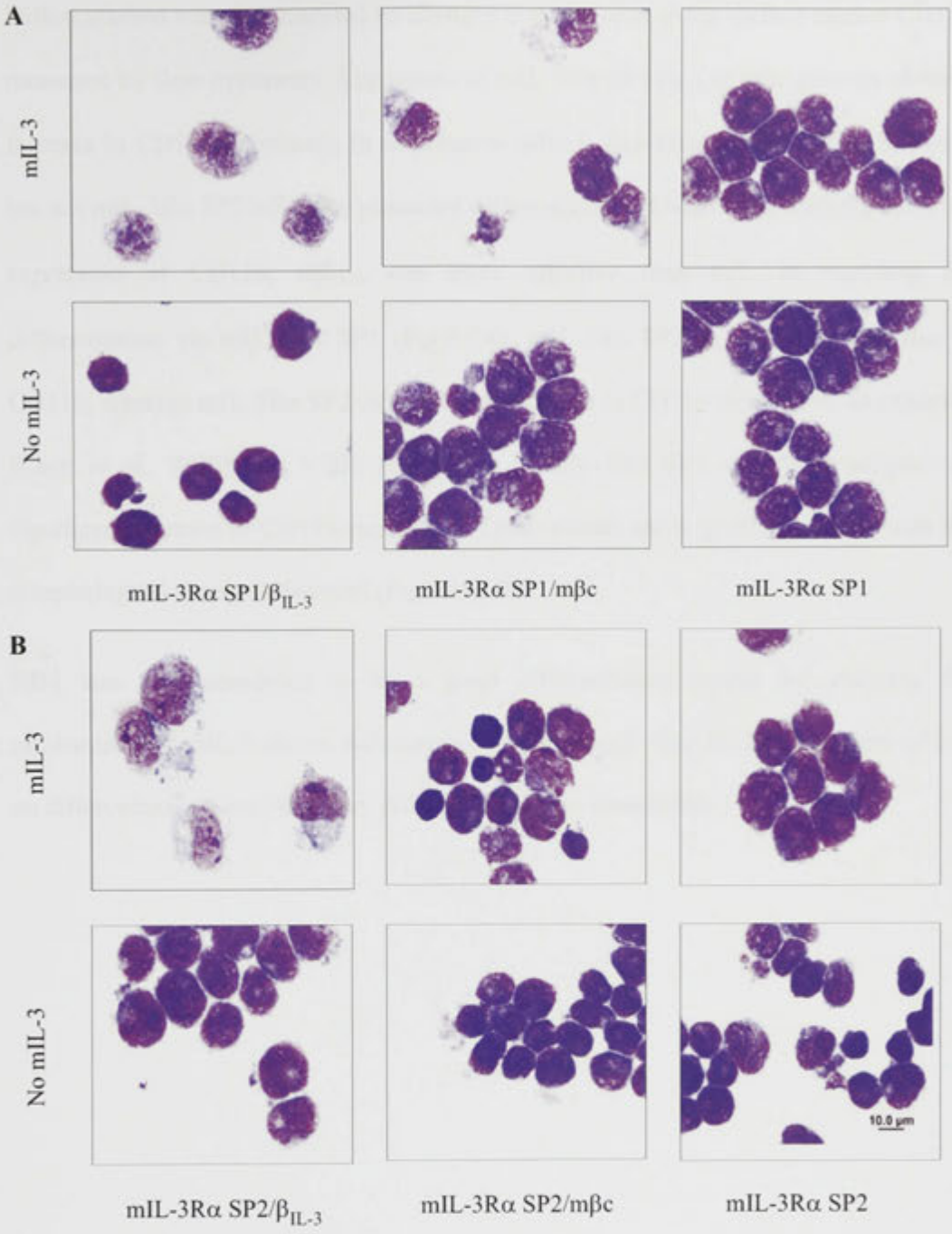


Figure 5.6: Morphological changes in NB4 cells expressing different mIL-3R subunits after 4 days culture with mIL-3. Responses to mIL-3 of NB4 cells expressing the mIL-3Rα SP1 or SP2 isoforms with and without $m\beta_{IL-3}$ or mβc after 4 days incubation were measured by changes in morphology. Morphological examination was made by light microscopy after May-Grunwald-Giemsa staining. Scale bar = 10 µm. Results represent one of three independent experiments.

Differentiation was also assessed by changes in expression of the surface marker CD11b measured by flow cytometry. Expression of mIL-3Rα SP1/β_{IL-3} or mβc gave an obvious increase in CD11b expression in response to mIL-3. Expression of mIL-3Rα SP1/mβc but not mIL-3Rα SP2/mβc also promoted differentiation of NB4 cells. Judging from the expression of CD11b, $m\beta_{IL-3}$ was more effective than mβc in signaling for differentiation via mIL-3Rα SP1 (Fig.5.7A). mIL-3Rα SP2/β_{IL-3} gave an increase in CD11b, whereas mIL-3Rα SP2/mβc gave no increase in CD11b expression, as expected (Chen *et al.*, 2009) (Fig.5.7B). Expression of mIL-3Rα SP1 or SP2 alone gave no significant increase in CD11b expression. These results are in good agreement with the morphological changes observed (Fig.5.6A, B).

NB4 was not considered to be a good differentiation model for studying the mechanisms of mIL-3-driven differentiation as, although clear IL-3R-dependent effects on differentiation were observed, differentiation was incomplete.

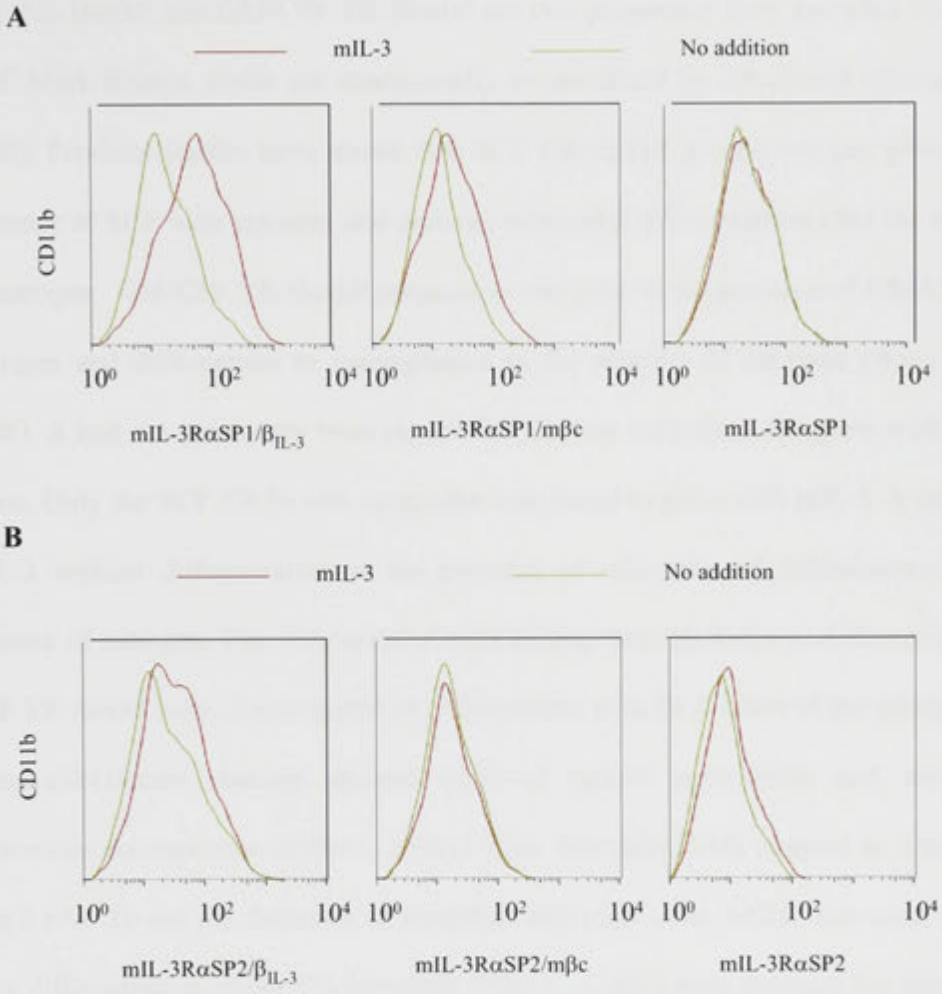


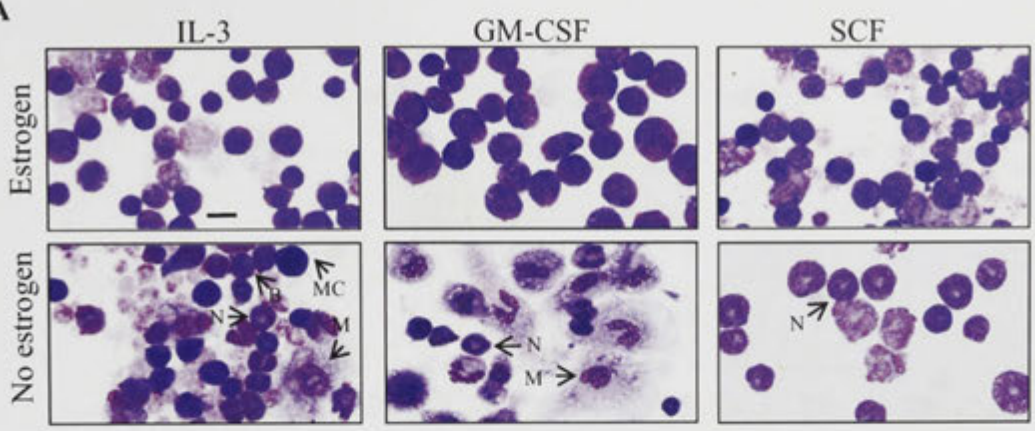
Figure 5.7: FACS analysis of expression of the surface marker CD11b. NB4 cells expressing the mIL-3R different subunits were cultured with or without mIL-3 for 4 days. Expression of CD11b in NB4 cells was measured by FACS as described in Methods. Data were acquired on a FACSort cytometer and analysed using FlowJo software. Results represent one of three independent experiments.

5.2.3 mIL-3-driven differentiation of the granulocyte-macrophage progenitor SCF ER-Hoxb8

SCF ER-Hoxb8 and GM-CSF ER-Hoxb8 are two progenitor lines provided kindly by Prof. Mark Kamps which are conditionally immortalized by ER-Hoxb8 (Wang *et al.*, 2006). Previous studies have shown that SCF ER-Hoxb8 progenitors can grow in the presence of SCF with estrogen and undergo neutrophil differentiation after the removal of estrogen. GM-CSF ER-Hoxb8 progenitors can grow in the presence of GM-CSF and estrogen and differentiate to macrophages in the absence of estrogen (Wang *et al.*, 2006). It had not previously been reported if the two cells lines can grow with mIL-3 alone. Only the SCF ER-Hoxb8 progenitor was found to grow with mIL-3. It grows on mIL-3 without differentiation in the presence of estrogen and differentiates in the absence of estrogen. The differentiated cells arising from IL-3-driven differentiation of SCF ER-hoxb8 were characterized in collaboration with Dr J. Chen of our group. May-Grunwald-Giemsa staining showed 65% of typical neutrophils and also 25% monocytes/macrophages (65%±4; 25%±3 from 100 cells fields counted in 3 samples) (Fig.5.8A). To aid the detection of basophils and mast cells, FACS was used. After 4 days differentiation, about 5% basophils (Mar-1$^+$, C-kit$^-$) were detected but these cells died rapidly and represented only about 1% of the non-adherent cells after 6 days differentiation. After 4 days differentiation, mast cells (Mar-1$^+$, C-kit$^+$) were also detected. The mast cells did not rapidly die in contrast to the basophils. Transmission electron microscopy was used to verify the identity of the basophils and mast cells (Fig.5.8B). In the absence of estrogen, SCF ER-Hoxb8 was shown to differentiate to 100% neutrophils when cultured with SCF and to 45% neutrophils and 55% macrophages (45%±5; 55%±5 from 100 cells fields counted in 3 samples) when cultured with GM-CSF (Fig.5.8A) in agreement with previously reported findings

(Wang *et al.,* 2006). The non-adherent progeny were analyzed by flow cytometry leaving any adherent macrophages behind. FACS analysis of the differentiation surface markers showed an obvious increase in Mac-1 (general myeloid differentiation antigen) expression and an increase in Gr-1(neutrophil differentiation antigen) in the absence of estrogen. F4/80 (macrophage differentiation antigen) showed no change or a slight decrease in the absence of estrogen (Fig.5.9). This indicates that both mIL-3 and mGM-CSF, in the absence of SCF, support the differentiation of SCF ER-Hoxb8 progenitors.

A



Neutrophil	65	45	100
Monocyte/Macrophage	25	55	0
Basophil-like	10	0	0

B

Basophil Mast cell Neutrophil

Figure 5.8: Morphological analysis of SCF ER-Hoxb8 progenitor cells after culturing 4 days without estrogen (differentiation) or with estrogen (growth). SCF ER-Hoxb8 cells were cultured in 1% (v/v) insect cell conditioned media containing mIL-3, mGM-CSF or mSCF under proliferation or differentiation conditions for 4 days. **A:** Analysis of cell morphology by light microscopy after May-Grunwald Giemsa staining. *B* basophil, *N* neutrophil, *M* macrophage, *MC* mast cell. Scale bar =10μm. **B:** Electron microscopy of the non-adherent SCF ER-Hoxb8 cells grown under differentiation conditions in 1% (v/v) insect cell conditioned media containing mIL-3 for 4 days. Results represent one of three independent experiments.

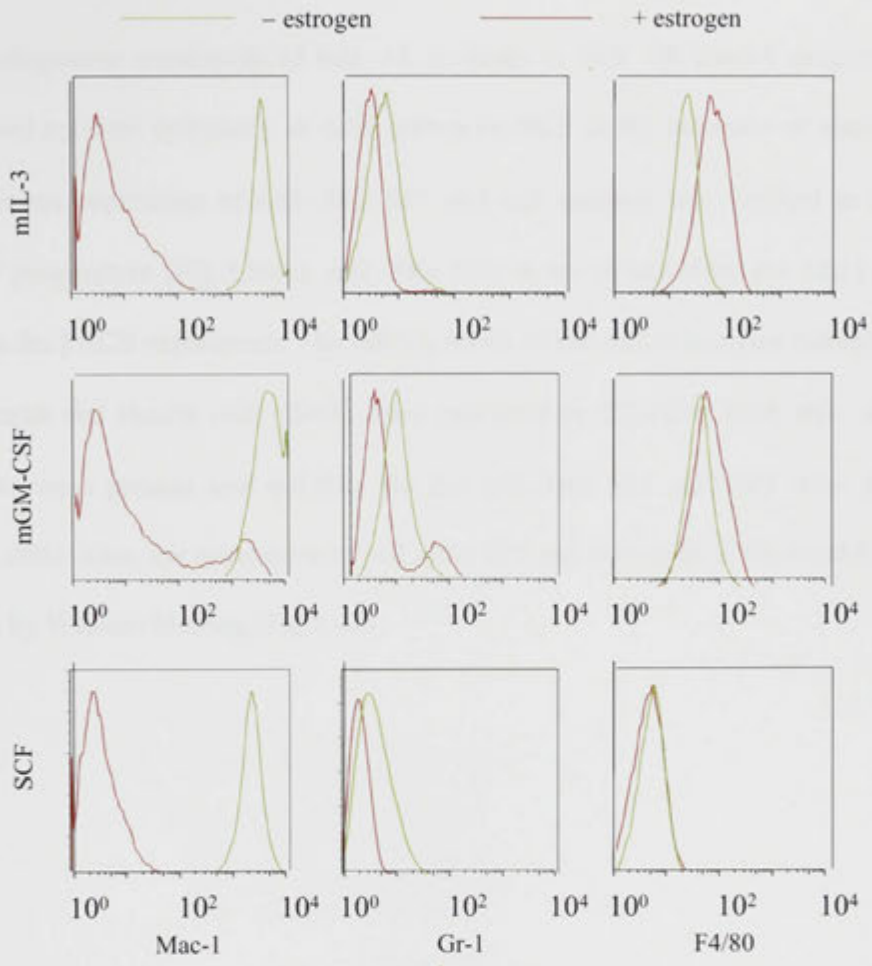


Figure 5.9: Surface marker expression of SCF ER-Hoxb8 progenitor cells after culturing 4 days without estrogen (differentiation) or with estrogen (growth). SCF ER-Hoxb8 cells were cultured in 1% (v/v) insect cell conditioned media containing mIL-3, mGM-CSF or SCF under proliferation or differentiation conditions for 4 days. FACS analysis of Mac-1 Gr-1, F4/80 expression on cells cultured with mIL-3, mGM-CSF or SCF without or with estrogen. Results represent one of three independent experiments.

5.2.4 Endogenous expression of mIL-3 receptors in SCF ER-Hoxb8 progenitors

The endogenous expression of mIL-3R subunits in SCF ER-Hoxb8 progenitors was measured by flow cytometry in cells grown on SCF in the presence of estrogen. The endogenous expression of mIL-3Rα SP1 and mβ receptor was verified in SCF ER-Hoxb8 progenitors (Fig.5.10A). mIL-3Rα SP2 is not detected by the 5B11 antibody used in the FACS experiment. The mRNA levels of the mIL-3 receptor subunits in SCF ER-Hoxb8 and Hoxb8 cells (B4.1) were measured by RT-PCR. Both mβc and $m\beta_{IL\text{-}3}$ mRNAs were present and mRNAs for the mIL-3Rα SP1 and SP2 were detectable (Fig.5.10B). Also, the expression of mIL-3Rα SP2 and SP1 in SCF ER-Hoxb8 cells was shown by Western blotting (Fig.5.11).

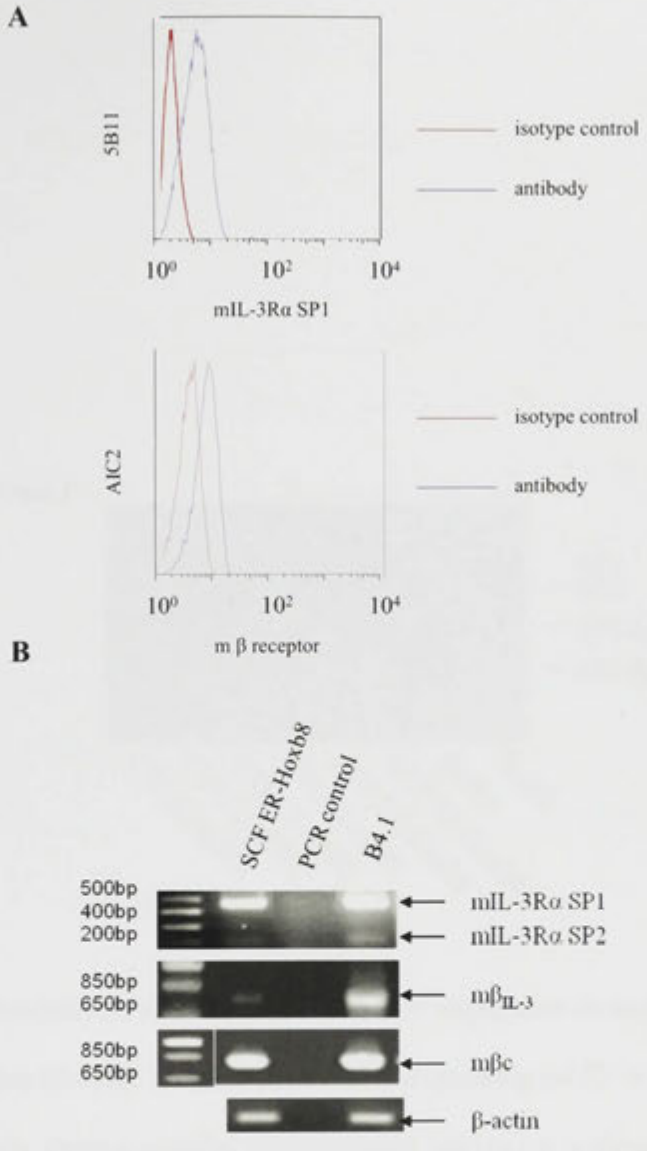


Figure 5.10: The endogenous expression of mIL-3R subunits in SCF ER-Hoxb8 cells. **A:** Detection of mIL-3Rα SP1 and mβ receptor in SCF ER-Hoxb8 cells by FACS. The red line histograms denote non-specific fluorescence obtained with the isotype control antibodies and are overlaid with the green line histograms obtained after labeling with 5B11 (rat anti-mouse IL-3Rα monoclonal antibody) or with the blue line labeling with AIC2 (rat anti-mouse β_{IL-3}/βc monoclonal antibody) and secondary reagent. **B:** mRNA levels of the mIL-3 receptors in Hoxb8 cells by RT-PCR. Both SP1 and SP2 isoforms of IL-3Rα were detectable with SP1 being the major form. Results represent one of at least two independent experiments.

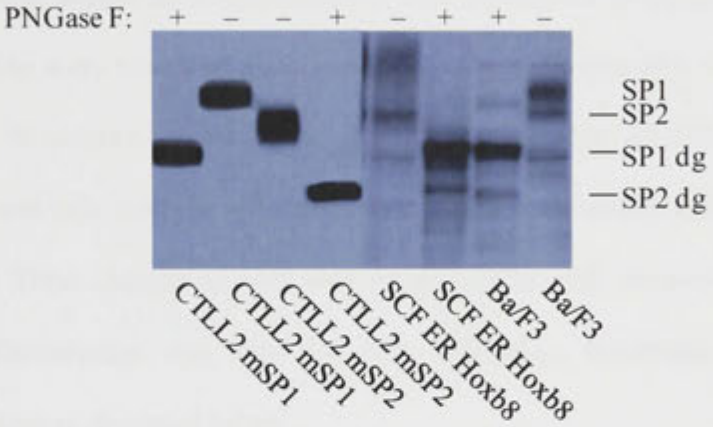


Figure 5.11: Analysis of mIL-3Rα SP2 and SP1 expression in mouse SCF ER-Hoxb8 cells by Western blotting. CTLL-2 cells stably expressing mSP2 or mSP1 were used as positive controls. Protein samples were analyzed with (+) or without (-) deglycosylation (dg) using peptide *N*-glycosidase F (*PNGase F*). Results represent one of at least two independent experiments.

5.2.5 mRNA levels of the mIL-3 and mGM-CSF receptor subunits in SCF ER-Hoxb8 progenitors undergoing IL-3- or GM-CSF-driven differentiation

SCF ER-Hoxb8 progenitors grow continuously on IL-3 or GM-CSF without differentiation in the presence of estrogen and differentiate in the absence of estrogen. With GM-CSF supported differentiation of SCF ER-Hoxb8, levels of mGM-CSFRα and mβc mRNAs were increased significantly whereas mIL-3Rα SP1 mRNA levels were decreased. In contrast, with mIL-3-supported differentiation of SCF ER-Hoxb8, mIL-3Rα SP1 and mβc mRNAs were decreased and $m\beta_{IL\text{-}3}$ mRNA levels were increased (Fig.5.12). These changes fit well with mGM-CSFRα /mβc involvement in GM-CSF-driven differentiation and with mIL-3Rα SP2/$m\beta_{IL\text{-}3}$ involvement in IL-3-driven differentiation as discussed below.

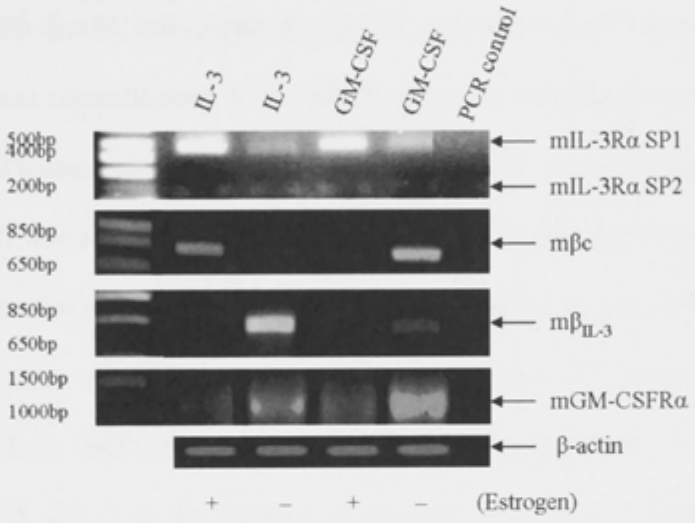


Figure 5.12: Measurement of the mRNA levels of the mIL-3 and mGM-CSF receptor subunits in SCF ER-Hoxb8 progenitors grown 4 days on IL-3 or GM-CSF with or without estrogen by RT-PCR. Results represent one of at least two independent experiments.

5.2.6 Sequencing of IL-3Rα cDNA clones isolated from SCF ER-Hoxb8 progenitors

cDNA clones of the mIL-3Rα isoforms were isolated from SCF ER-Hoxb8 progenitors cultured with mIL-3 in the presence or absence of estrogen using primers at the N-terminal and C-terminal of the coding sequence (Fig.5.13). Nine clones were sequenced from SCF ER-Hoxb8 cells cultured with mIL-3 in the presence of estrogen. Four clones had sequences corresponding to the SP1 isoform and two clones to the SP2 isoform and the encoded amino acid (aa) sequences of the isoforms were identical with the reference sequences (Chen *et al.,* 2009). Thus there was good expression of both IL-3Rα isoforms with no mutations in SCF ER-Hoxb8 cells. In addition to the canonical SP1 and SP2 sequences, three other cDNA clones carried mutations (197 insert 90bp; 353-385base deletion (11aa); 199 insert 33bp). Of the cDNA clones from SCF ER-Hoxb8 cells cultured with mIL-3 in the absence of estrogen, two clones corresponded to the SP1 isoform and three clones to the SP2 isoform with no mutations detected. Apart from these clones, three cDNAs carried point mutations (287 C→T, aa P→S; 836 G→A, aa A→T; 620 C→T, aa H→Y) and one carried a deletion of 353-385 base (11aa). The DNA sequencing of cDNA clones from SCF ER-Hoxb8 established that there were good levels of mRNAs encoding normal mIL-3Rα SP1 and SP2 isoforms during both growth and differentiation.

```
mIL-3Rα SP2          MAANLWLILGLLASHSSDL------------------------------   19
mIL-3Rα SP1          MAANLWLILGLLASHSSDLAAVREAPPTAVTTPIQNLHIDPAHYTLSWDP   50
REF mIL-3Rα SP1      MAANLWLILGLLASHSSDLAAVREAPPTAVTTPIQNLHIDPAHYTLSWDP   50
REF mIL-3Rα SP2      MAANLWLILGLLASHSSDL------------------------------   19

mIL-3Rα SP2          ---------------------------------------------------   19
mIL-3Rα SP1          APGADITTGAFCRKGRDIFVWADPGLARCSFQSLSLCHVTNFTVFLGKDR  100
REF mIL-3Rα SP1      APGADITTGAFCRKGRDIFVWADPGLARCSFQSLSLCHVTNFTVFLGKDR  100
REF mIL-3Rα SP2      ---------------------------------------------------   19

mIL-3Rα SP2          ------------DGDHEAAAQDLRCWVHEGQLSCQWERGPKATGDVHYRM   57
mIL-3Rα SP1          AVAGSIQFPPDDDGDHEAAAQDLRCWVHEGQLSCQWERGPKATGDVHYRM  150
REF mIL-3Rα SP1      AVAGSIQFPPDDDGDHEAAAQDLRCWVHEGQLSCQWERGPKATGDVHYRM  150
REF mIL-3Rα SP2      ------------DGDHEAAAQDLRCWVHEGQLSCQWERGPKATGDVHYRM   57

mIL-3Rα SP2          FWRDVRLGPAHNRECPHYHSLDVNTAGPAPHGGHEGCTLDLDTVLGSTPN  107
mIL-3Rα SP1          FWRDVRLGPAHNRECPHYHSLDVNTAGPAPHGGHEGCTLDLDTVLGSTPN  200
REF mIL-3Rα SP1      FWRDVRLGPAHNRECPHYHSLDVNTAGPAPHGGHEGCTLDLDTVLGSTPN  200
REF mIL-3Rα SP2      FWRDVRLGPAHNRECPHYHSLDVNTAGPAPHGGHEGCTLDLDTVLGSTPN  107

mIL-3Rα SP2          SPDLVPQVTITVNGSGRAGPVPCMDNTVDLQRAEVLAPPTLTVECNGSEA  157
mIL-3Rα SP1          SPDLVPQVTITVNGSGRAGPVPCMDNTVDLQRAEVLAPPTLTVECNGSEA  250
REF mIL-3Rα SP1      SPDLVPQVTITVNGSGRAGPVPCMDNTVDLQRAEVLAPPTLTVECNGSEA  250
REF mIL-3Rα SP2      SPDLVPQVTITVNGSGRAGPVPCMDNTVDLQRAEVLAPPTLTVECNGSEA  157

mIL-3Rα SP2          HARWVARNRFHHGLLGYTLQVNQSSRSEPQEYNVSIPHFWVPNAGAISFR  207
mIL-3Rα SP1          HARWVARNRFHHGLLGYTLQVNQSSRSEPQEYNVSIPHFWVPNAGAISFR  300
REF mIL-3Rα SP1      HARWVARNRFHHGLLGYTLQVNQSSRSEPQEYNVSIPHFWVPNAGAISFR  300
REF mIL-3Rα SP2      HARWVARNRFHHGLLGYTLQVNQSSRSEPQEYNVSIPHFWVPNAGAISFR  207
```

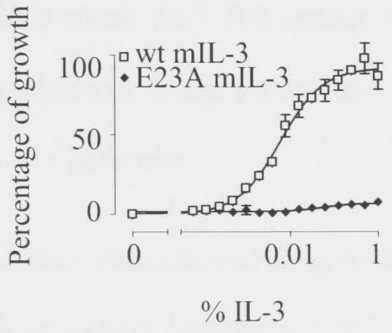
Figure 5.13: Amino acid sequences of mIL-3Rα isoforms encoded by cDNA clones derived from SCF ER-Hoxb8 cells grown for 4 days with mIL-3 and with and without estrogen. REF mIL-3Rα SP1 and SP2 (Chen *et al.*, 2009) are the previously reported reference sequences for these receptor subunits.

5.2.7 IL-3 signaling pathways during self-renewal and differentiation in SCF ER-Hoxb8 progenitors

5.2.7.1 Utilization of IL-3Rα isoforms by SCF ER-Hoxb8

In other research in our group, a mIL-3 mutant has been characterized which has near wild type activity with the IL-3Rα SP1 isoform and no activity with the SP2 isoform (Mirza *et al.*, 2010). This mutant, mIL-3 E23A, provides a test for the critical utilization of the SP2 isoform. In collaboration with S. Mirza of our group, the utilization of the IL-3Rα SP2 isoform by SCF ER-Hoxb8 was measured using mIL-3 E23A. Interestingly, SCF ER-Hoxb8 grew very poorly on mIL-3 E23A in comparison with the IL-3-dependent cell line Ba/F3 (Fig.5.14). mIL-3 E23A also did not effectively drive differentiation of SCF ER-Hoxb8. This is despite IL-3Rα SP1 being the major isoform expressed in these cells. Since the levels of IL-3Rα SP1 expressed in Ba/F3 and SCF ER-Hoxb8 are similar, it is likely that a specific mechanism is present in SCF ER-Hoxb8 blocking signaling via IL-3Rα SP1. This could facilitate differentiation signaling via IL-3Rα SP2 in GM progenitors.

A



B

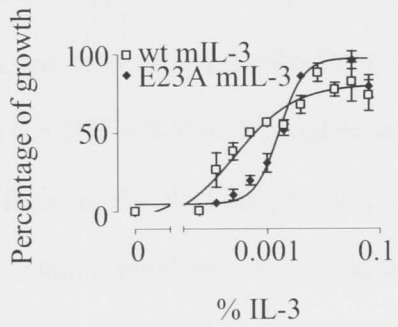


Figure 5.14: Growth assay of cells cultured with wild type mIL-3 or mIL-3 E23A. **A:** SCF ER-Hoxb8 cells grown with estrogen; **B:** Ba/F3 cells. Cells were grown in media supplemented with various percentages (v/v) of insect cell conditioned media containing either wild type mIL-3 or mIL-3 E23A as indicated for 2 days, and percentage of growth was measured in triplicate by [^{3}H]-thymidine incorporation. Results represent one of at least two independent experiments.

5.2.7.2 IL-3 signaling pathways during growth and differentiation in SCF ER-Hoxb8

Studies of the signaling pathways linked to the mIL-3R have primarily been carried out with cell lines like Ba/F3 which don't differentiate. It was of interest to compare the known IL-3 signaling pathways during growth and differentiation and to use mIL-3 E23A to test mIL-3Rα SP1 utilization.

SCF ER-Hoxb8 progenitors were allowed to grow or differentiate by culturing with mIL-3 for 4 days with or without estrogen and the non-adherent cells were collected. The cells were starved of IL-3 and then stimulated for 10 minutes with mIL-3 or mIL-3 E23A and then lysed. The lysates were subjected to SDS-PAGE and immunoblotted using phosphospecific and total protein antibodies to Jak2, STAT5, Erk1/2, Akt. IL-3 signaling pathways in the SCF ER-Hoxb8 progenitors during growth or differentiation were compared with those in the IL-3-dependent cell line Ba/F3 during growth. The janus activated kinase (Jak) /signal transducer and activator of transcription (STAT) pathway plays a critical role for cytokine receptor-mediated intracellular signaling and hematopoietic cell development (Nosaka and Kitamura, 2000). The extracellular signal-regulated kinase 1/2 (Erk1/2), is a serine/threonine kinase that appears to be one of the key regulators of cell proliferation and differentiation (McCubrey *et al.*, 2000; Lee and McCubrey, 2002). Akt is a protein serine/threonine kinase and the major effector of the PI3-K pathway. It was found that Jak2/STAT5, Akt and Erk1/2 were activated efficiently in SCF ER-Hoxb8 progenitors treated with mIL-3, but not in cells treated with mIL-3 E23A.

In agreement with the growth data presented above (Fig.5.14), Ba/F3 cells showed no measurable differences in Jak2 and Erk1/2 signaling when stimulated with wild-type

mIL-3 or mIL-3 E23A. In contrast, SCF ER-Hoxb8 cells showed a dramatic reduction in Jak2 and Erk activation when stimulated with mIL-3 E23A. This indicates that IL-3 signaling in SCF ER-Hoxb8 is predominantly via IL-3Rα SP2 whereas IL-3Rα SP1 is utilized by Ba/F3 cells (Fig.5.15). Interestingly, the differentiated progeny cells could utilize IL-3Rα SP1 for signaling via Erk1/2 (Fig.5.15C).

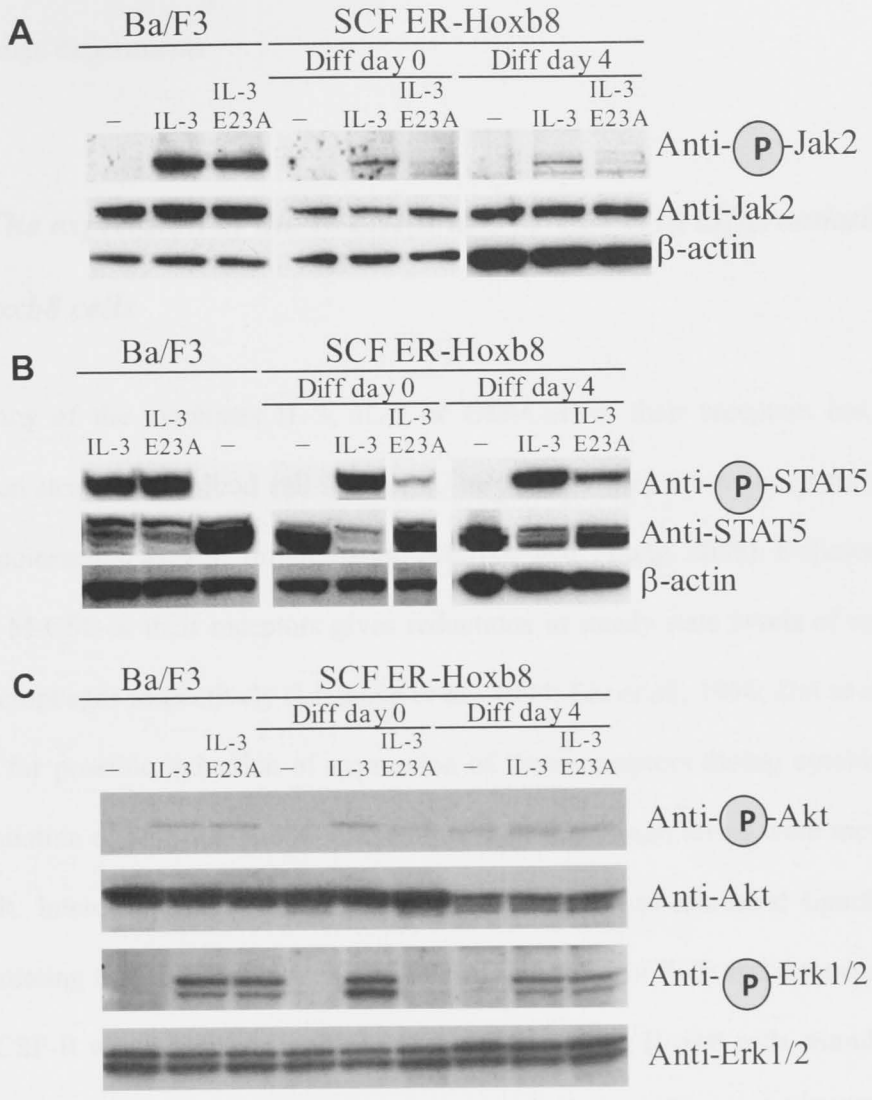


Figure 5.15: Activation of Jak2, STAT5, AKT and Erk1/2 signaling in Ba/F3 and SCF ER-Hoxb8 cells by wild-type and mIL-3 E23A. BaF/3 cells and SCF ER-Hoxb8 cells cultured in 1% (v/v) insect cell conditioned media containing wild type mIL-3 or SCF respectively were starved for 4 h before stimulation with wild-type mIL-3, mIL-3 E23A , or no (–) mIL-3 for 10 min. The differentiated SCF ER-Hoxb8 cells were cultured in 1% (v/v) insect cell conditioned media containing wild type mIL-3 without estrogen for 4 days. Western blots were performed to detect phospho-Jak2 ($Tyr^{1007/1008}$), total Jak2, phospho-STAT5 (Tyr^{691}), total STAT5, phospho-Akt (Ser^{473}), total Akt, phospho-

Erk1/2 (Thr^{202}/Tyr^{204}) and total Erk1/2. Results were verified in at least three independent experiments.

5.2.8 The expression of the G-CSFR and M-CSFR in differentiating SCF ER-Hoxb8 cells

Deficiency of the cytokines IL-3, IL-5 or GM-CSF or their receptors has minimal effects on steady state blood cell formation. In contrast, they are required for inducible hematopoiesis and allergic inflammation (Murphy and Young, 2006). Deficiency of G-CSF or M-CSF or their receptors gives reductions in steady-state levels of neutrophils and macrophages respectively (Lieschke *et al.,* 1994; Liu *et al.,* 1996; Dai *et al.,* 2002). To test for possible induction of expression of these receptors during cytokine-driven differentiation of SCF ER-Hoxb8 progenitors their expression levels were measured by RT-PCR. Interestingly, the expression of the G-CSF-R was increased significantly in differentiating SCF ER-Hoxb8 cells stimulated with IL-3 or SCF and the expression of the M-CSF-R was increased significantly in differentiating Hoxb8 cells stimulated with GM-CSF (Fig.5.16). The patterns of expression of the G-CSFR and M-CSFR fits well with the role of these receptors in the steady state differentiation pathways to granulocytes and monoctyes.

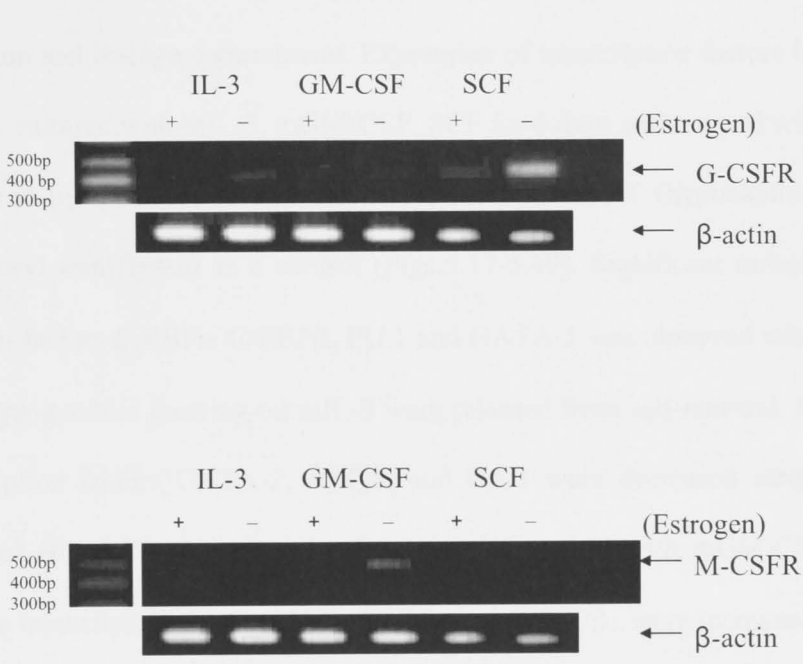


Figure 5.16: Measurement of expression of the G-CSF receptor and M-CSF receptors in SCF ER-Hoxb8 progenitors cultured with mouse IL-3, GM-CSF or SCF for 4 days with and without estrogen using RT-PCR. Results represent one of at least two independent experiments.

5.2.9 Involvement of transcription factors and miRNAs in the switch between proliferation and differentiation

5.2.9.1 Induced changes in transcription factor expression

Transcription factors are known to play a major role in regulating hematopoietic differentiation and lineage commitment. Expression of transcription factors in SCF ER-Hoxb8 cells cultured with mIL-3, mGM-CSF, SCF for 4 days without and with estrogen was measured by quantitative real time RT-PCR with HPRT (hypoxanthine-guanine phosphoribosyl transferase) as a control (Figs.5.17-5.19). Significant induction of the transcription factors C/EBPε, C/EBPβ, PU.1 and GATA-1 was observed when the SCF ER-Hoxb8 progenitors growing on mIL-3 were released from self-renewal. In addition, the transcription factors GATA-2, c-Myb, and Gfi-1 were decreased when the cells differentiated (Fig.5.17). In cells stimulated to differentiate with mGM-CSF, mRNA levels of the transcription factors PU.1, C/EBPε and C/EBPβ, were increased and those for c-Myb were decreased (Fig.5.18). In cells stimulated to differentiate with SCF, up-regulation of C/EBPε, PU.1 and GATA-1 and down-regulation of GATA-2, c-Myb, and Gfi-1 was observed (Fig.5.19). A comparison was also made of the changes of transcription factors using non-adherent or adherent cells stimulated to differentiate with IL-3 or GM-CSF for 2 days. C/EBPε was elevated significantly in non-adherent cells with IL-3 (Table 5.2).

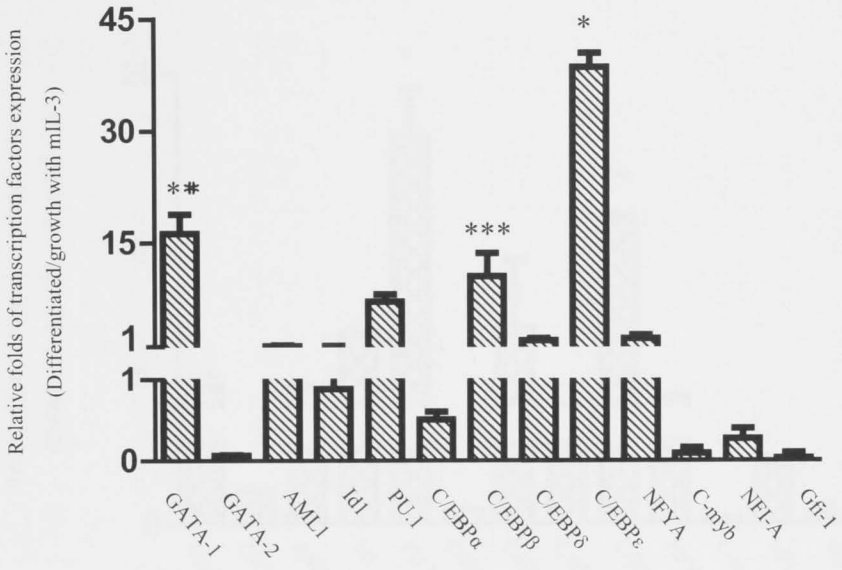


Figure 5.17: Expression of transcription factors in SCF ER-Hoxb8 cells cultured with mIL-3 for 4 days without and with estrogen by real time RT-PCR. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen). Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.0001$; **, $p=0.0005$; ***, $p=0.0054$ by student's t test.

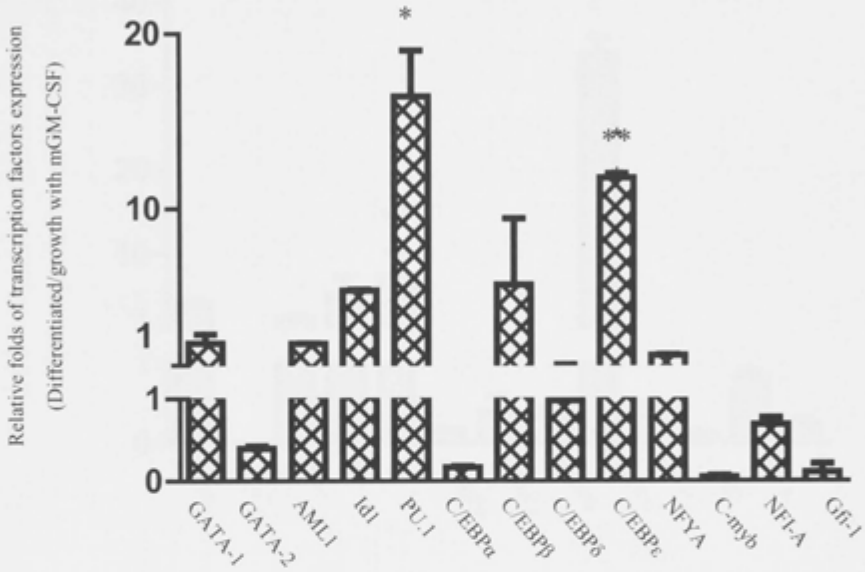


Figure 5.18: Expression of transcription factors in SCF ER-Hoxb8 cells cultured with mGM-CSF for 4 days without and with estrogen by real time RT-PCR. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen). Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.001$; **, $p<0.001$ by student's *t* test.

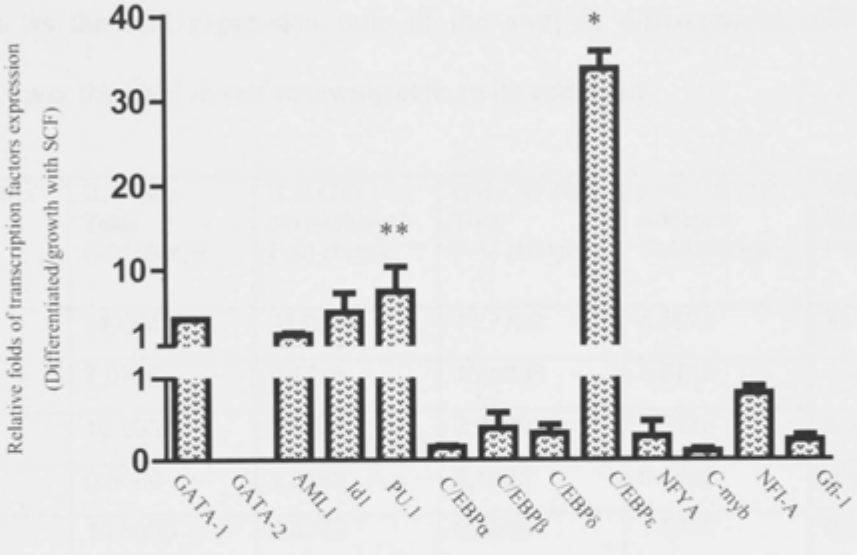


Figure 5.19: Expression of transcription factors in SCF ER-Hoxb8 cells cultured with SCF for 4 days without and with estrogen by real time RT-PCR. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen). Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.0001$; **, $p<0.01$ by student's t test.

Table 5.2: Relative fold changes in transcription factor expression in ER-Hoxb8 cells cultured with IL-3, GM-CSF or SCF for 4 days or 2 days without estrogen (adherent, non-adherent or total cells) and with estrogen. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen).

Transcription factor	IL-3 (4d) Total Fold change	IL-3 (2d) Non-adherent Fold change	GM-CSF (4d) Total Fold change	GM-CSF (2d) Adherent Fold change	SCF (4d) Total Fold change
C/EBPε	38.6447	31.0506	11.7732	4.7672	33.7742
PU.1	7.0758	2.7715	16.4308	3.6718	7.3387
GATA-1	16.2507	3.5774	2.2830	5.5170	4.1363
C/EBPα	0.5080	1.2588	0.1623	0.5485	0.1597
C/EBPβ	10.5095	0.4772	5.6356	1.5390	0.3829
C/EBPδ	1.7985	0.7868	0.9755	1.1779	0.3199
Id1	0.8831	1.8357	5.3135	1.4727	4.8688
NFI-A	0.2694	0.4354	0.6807	0.8703	0.8079
Gfi-1	0.0314	0.9490	0.1068	0.7702	0.2343
GATA-2	0.0657	0.2280	0.3962	0.2402	0.0004
c-Myb	0.0886	0.6685	0.0452	0.3878	0.0999
AML1	1.0622		2.2618		2.2373
NFYA	2.0542		1.5695		0.2865

5.2.9.2 Induced changes in expression of miRNAs

miRNAs regulate the translation and stability of specific mRNAs and are believed to significantly contribute to the regulation of lineage commitment and differentiation (Fatica *et al.,* 2008). Initially, the levels of 11 miRNAs known to be involved in the regulation of differentiation and hematopoiesis were measured by real-time PCR in SCF ER-Hoxb8 cells triggered to differentiate. In cells undergoing differentiation supported by either IL-3, GM-CSF or SCF, miR-223 was highly induced. The changes in miR-223 during the time course of differentiation supported by IL-3 showed that miR-223 increased gradually from 1 day to 4 days in the absence of estrogen (Fig.5.21). This is in agreement with a number of reports implicating miR-223 in the functional maturation of granulocytes (Fazi *et al.,* 2005; Fatica *et al.,* 2006; Johnnidis *et al.,* 2008). In contrast, the levels of miR-155, miR-181a and miR-292 were decreased in differentiating cells grown in IL-3 or GM-CSF and miR-155 was decreased in differentiating cells grown in SCF (Figs.5.20, 5.22 and 5.23).

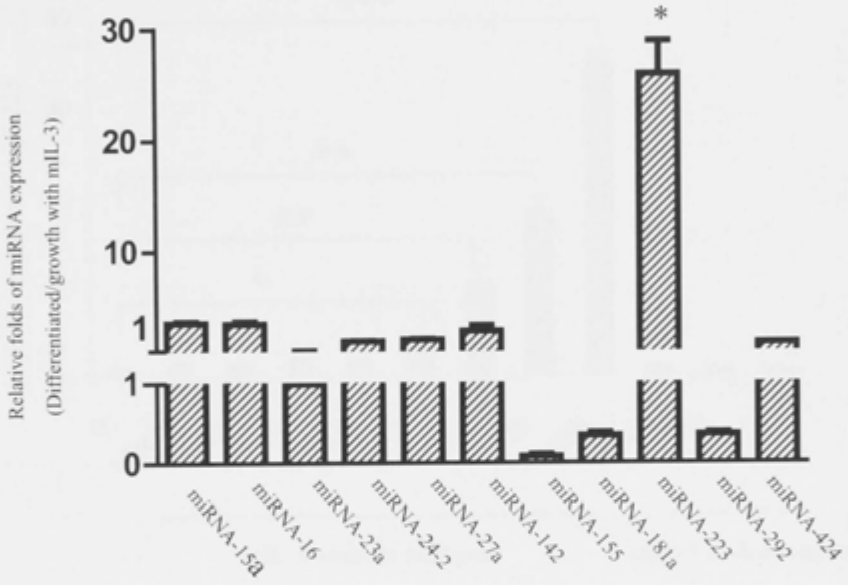


Figure 5.20: Expression of miRNAs in ER-Hoxb8 cells cultured with mIL-3 for 4 days without and with estrogen by real time RT-PCR. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen). Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.0001$ by student's t test.

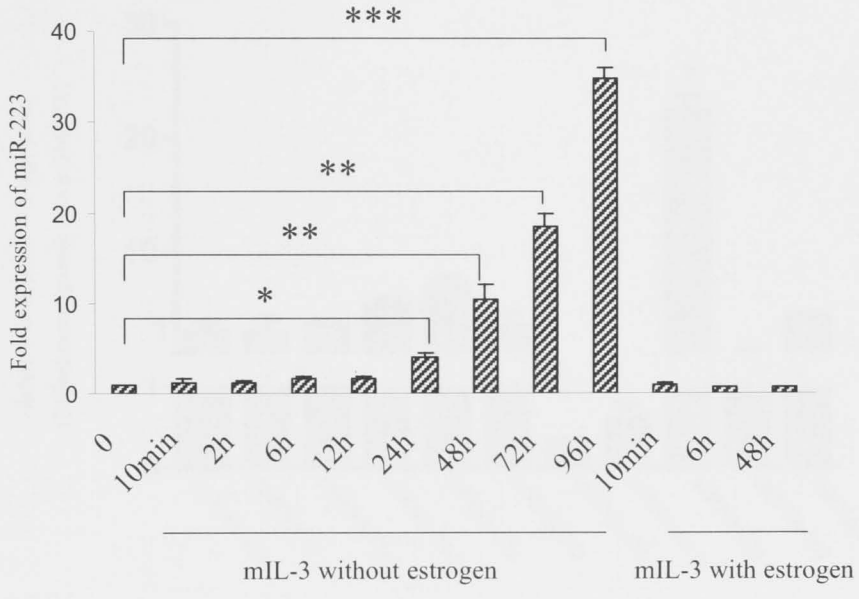


Figure 5.21: Time course of expression of miR-223 in SCF ER-Hoxb8 cells cultured with mIL-3 without and with estrogen. The fold expression of all samples is relative to the control RNA which is labelled "0". Results were verified in at least two independent experiments. Data are mean, error bars are standard deviations. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ by student's t test.

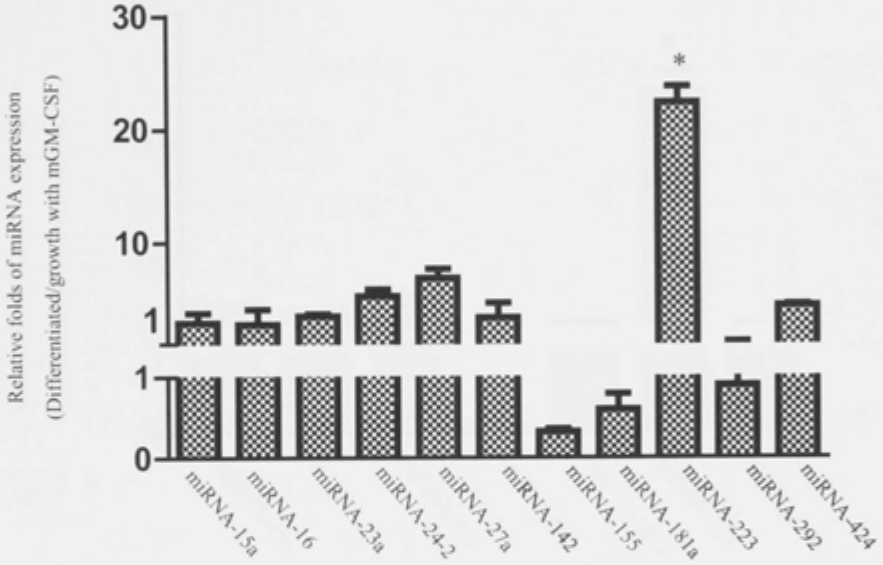


Figure 5.22: Expression of miRNAs in ER-Hoxb8 cells cultured with mGM-CSF for 4 days without and with estrogen by real time RT-PCR. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen). Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.0001$ by student's t test.

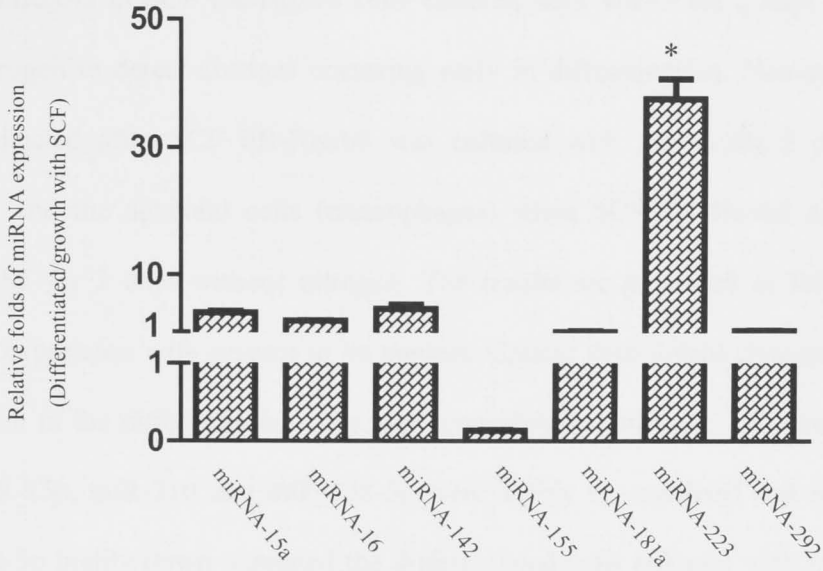


Figure 5.23: Expression of miRNAs in ER-Hoxb8 cells cultured with SCF for 4 days without and with estrogen by real time RT-PCR. Expression of each component is presented as the fold expression ratio of the level in differentiated cells (without estrogen) over the level in self-renewing cells (with estrogen). Results were verified in three independent experiments. Data are mean, error bars are standard deviations. *, $p<0.0001$ by student's t test.

5.2.10 MiRNA-mediated molecular circuitries in granulocytic differentiation

Analysis of miRNA expression is an important step in elucidating miRNA function. The Affymetrix miRNA microarray platform was also used to compare the expression of mouse miRNAs in SCF ER-Hoxb8 cells cultured with mIL-3 for 2 days without and with estrogen to detect changes occurring early in differentiation. Non-adherent cells were collected after SCF ER-Hoxb8 was cultured with mIL-3 for 2 days without estrogen and the adherent cells (macrophages) when SCF ER-Hoxb8 cultured with mGM-CSF for 2 days without estrogen. The results are presented as fold change of miRNA expression with respect to its control. Greater than 2-fold changes in miRNA expression in the differentiating cells were considered significant. In addition to miR-223, miR-15a, miR-210 and miR-338-5p were highly up-regulated and miR-708 and miR-324-3p highly down-regulated the differentiated cells cultured with mIL-3 (Table 5.3). Whereas miR-210, miR-139-3p, miR-423-5p and miR-200 were highly up-regulated and miR-466-5p, miR-421 and miR-708 highly down-regulated in macrophages cultured with mGM-CSF (Table 5.4). miR-210, miR-223, miR-339-5p were up-regulated and miR-708, miR-181d were down-regulated both in the cells differentiated with mIL-3 and macrophages differentiated with mGM-CSF.

Table 5.3: Relative fold expression of miRNAs in ER-Hoxb8 cells cultured with mIL-3 for 2 days without and with estrogen. Affymetrix miRNA microarray analysis of the expression of 609 mouse miRNAs. Raw data were normalized after background subtraction using global median of the intensities. In the histograms, the results are presented as above 2-fold change of miRNA expression with respect to the control (growth with estrogen).

Upregulated microRNA (-estrogen/+estrogen)	Fold change	Downregulated microRNA (-estrogen/+estrogen)	Fold change
mmu-miR-15a-star	10.0813	mmu-miR-708	-14.9411
mmu-miR-210	7.41527	mmu-miR-324-3p	-7.01336
mmu-miR-338-5p	6.76612	mmu-miR-146b-star	-4.51752
mmu-miR-223	6.74026	mmu-miR-139-3p	-4.45189
mmu-miR-466a-3p	4.13576	mmu-miR-669a	-4.43455
mmu-let-7b-star	3.50846	mmu-miR-301b	-4.35723
mmu-miR-488	3.04903	mmu-let-7g-star	-4.10465
mmu-miR-297a	2.92813	mmu-let-7d-star	-4.03176
mmu-miR-494	2.90112	mmu-miR-146a	-3.87895
mmu-miR-16-star	2.77259	mmu-miR-30e	-3.79337
mmu-miR-883b-3p	2.74271	mmu-miR-34b-3p	-3.5051
mmu-miR-466j	2.63969	mmu-miR-155	-3.23504
mmu-miR-148a-star	2.44478	mmu-miR-27a-star	-3.14742
mmu-miR-615-5p	2.41843	mmu-miR-330-star	-3.14452
mmu-miR-325-star	2.41275	mmu-miR-877	-3.11208
mmu-miR-15b	2.33739	mmu-miR-467g_st	-3.06459
mmu-miR-133b	2.29989	mmu-miR-181a-1-star	-2.96668
mmu-miR-669h-3p	2.29897	mmu-miR-295-star	-2.96263
mmu-miR-92a-star	2.28157	mmu-miR-194	-2.93591
mmu-miR-192	2.27768	mmu-let-7f	-2.92428
mmu-miR-483-star	2.25807	mmu-miR-296-5p	-2.87554
mmu-miR-504	2.23538	mmu-miR-1192	-2.66171
mmu-miR-322	2.22399	mmu-miR-181d	-2.66007
mmu-miR-335-5p	2.20422	mmu-miR-486	-2.655
mmu-miR-151-3p	2.17377	mmu-miR-467e-star	-2.62041
mmu-miR-199b-star	2.15404	mmu-miR-378-star	-2.61495
mmu-miR-466f-3p	2.10006	mmu-miR-345-5p	-2.34548
mmu-miR-302b-star	2.09659	mmu-miR-503	-2.24069
mmu-miR-374-star	2.0833	mmu-miR-487b	-2.17616
mmu-miR-153	2.07073	mmu-miR-712-star	-2.10372
mmu-miR-342-3p	2.04919	mmu-miR-140-star	-2.0355
mmu-miR-339-5p	2.01439	mmu-miR-200b-star	-2.01638
mmu-miR-677	2.01046	mmu-miR-345-3p	-2.00294
mmu-miR-16	2.00164	mmu-miR-874	-2.00089
mmu-miR-770-3p_	2.00078		

Table 5.4: Relative fold expression of miRNAs in ER-Hoxb8 cells cultured with mGM-CSF for 2 days without and with estrogen.

Upregulated microRNA (-estrogen/+estrogen)	Fold change	Downregulated microRNA (-estrogen/+estrogen)	Fold change
mmu-miR-210	67.1135	mmu-miR-466f-5p	-38.8015
mmu-miR-139-3p	24.9937	mmu-miR-421	-38.0426
mmu-miR-423-5p	11.9852	mmu-miR-708	-37.113
mmu-miR-200c	11.6448	mmu-miR-29a	-30.7649
mmu-miR-700	7.34154	mmu-miR-669c	-24.4433
mmu-miR-339-5p	6.47265	mmu-miR-1187	-18.8575
mmu-miR-149	6.11887	mmu-miR-27b-star	-18.7192
mmu-miR-320	5.63614	mmu-miR-183	-15.7939
mmu-miR-744	4.60775	mmu-miR-181d	-12.4855
mmu-miR-503	4.53228	mmu-miR-466c-5p	-12.4426
mmu-miR-351	4.34515	mmu-miR-466f	-12.1067
mmu-miR-877	4.24376	mmu-miR-706	-8.98144
mmu-miR-324-5p	4.24234	mmu-miR-674-star	-8.91225
mmu-miR-411	3.91161	mmu-miR-466j	-8.7236
mmu-miR-330-star	3.82128	mmu-miR-34a	-8.68018
mmu-miR-197	3.71742	mmu-miR-466h	-8.62144
mmu-miR-140-star	3.64369	mmu-miR-19a	-8.48764
mmu-miR-423-3p	3.6305	mmu-let-7g	-7.61579
mmu-miR-298	3.59883	mmu-miR-466c-3p	-6.52831
mmu-miR-484	3.55326	mmu-miR-155	-6.34483
mmu-miR-702	3.54145	mmu-miR-15a	-5.90134
mmu-miR-185	3.51897	mmu-miR-669b	-5.84019
mmu-miR-223	3.39345	mmu-miR-466b-3p	-5.8078
mmu-miR-182	3.08227	mmu-miR-1224	-5.01183
mmu-miR-674	2.94435	mmu-miR-467c	-4.79775
mmu-miR-106b-star	2.47348	mmu-miR-425-star	-4.31763
mmu-miR-296-3p	2.45936	mmu-miR-345-3p	-4.18605
mmu-miR-103	2.42065	mmu-miR-93-star	-4.06823
mmu-miR-467e-star	2.41869	mmu-let-7i	-3.83814
mmu-miR-17-star	2.40776	mmu-let-7f	-3.71558
mmu-miR-652	2.35986	mmu-miR-466e-3p	-3.54537
mmu-miR-27a-star	2.18406	mmu-miR-20b	-3.43348
mmu-miR-215	2.13602	mmu-miR-27b	-3.37576
mmu-miR-532-5p	2.13554	mmu-miR-25	-3.29103
mmu-miR-720	2.01579	mmu-miR-714	-3.16664
		mmu-miR-297a	-3.13486
		mmu-miR-467a	-2.95292
		mmu-miR-378-star	-2.92468
		mmu-miR-183-star	-2.70655
		mmu-let-7a	-2.56126
		mmu-miR-19b	-2.52892
		mmu-miR-466a-3p	-2.50725
		mmu-miR-106a	-2.42859
		mmu-miR-194	-2.36924
		mmu-miR-15a-star	-2.16935
		mmu-miR-140	-2.15724
		mmu-miR-574-5p	-2.07455
		mmu-miR-181a-1-star	-2.06184

5.3 Discussion

As part of a search for a model to study mIL-3-driven myeloid differentiation, three differentiation models were investigated. Ectopic expression of mIL-3R subunits in M1 mouse myeloblastic leukemia cells or NB4 human promyelocytic leukemia cells gave only partial differentiation in response to mIL-3. A far superior model was SCF ER-Hoxb8 progenitors which grow on mIL-3 or mGM-CSF without differentiation in the presence of estrogen and differentiate essentially completely in its absence. The SCF ER-Hoxb8 progenitor line therefore provides an excellent model to study the mechanisms controlling differentiation and self-renewal.

Deficiency of G-CSF or M-CSF or their receptors gives reductions in steady-state levels of neutrophils and macrophages respectively (Lieschke *et al.,* 1994; Liu *et al.,* 1996; Dai *et al.,* 2002). Thus these receptors are involved in steady state hematopoiesis and the steady state differentiation pathway. It was therefore of interest to see if these receptors were expressed during the cytokine-induced differentiation of SCF ER-Hoxb8 progenitors. The results showed that the G-CSFR is induced during differentiation in IL-3 and SCF and the M-CSFR is induced during differentiation with GM-CSF. This represents an additional similarity between the cytokine-induced and steady state myeloid differentiation pathways.

Our group has recently discovered a new isoform of the IL-3Rα (designated SP2), which is expressed by mouse and human hematopoietic cells (Chen *et al.,* 2009). Recent structure-function studies have shown there are distinct epitopes on IL-3 and the β receptors which are utilized by mIL-3Rα SP1 and SP2 isoforms (Mirza *et al.,* 2010). It seems likely that that the two high affinity complexes involve different relative orientations of the receptor subunits resulting in different signaling properties. An IL-3

mutant, mIL-3 E23A, has been characterized by our group which supports IL-3 growth signaling via the IL-3Rα SP1 isoform but not via the SP2 isoform. In the work described in this Chapter it was shown that SCF ER-Hoxb8 progenitors did not grow with the mutant mIL-3. This indicates that the cell line predominantly uses the IL-3Rα SP2 isoform for mIL-3 signaling. Sequencing analysis and Western blotting showed that both IL-3Rα SP1 and SP2 isoforms are expressed in SCF ER-Hoxb8. Indeed the SP1 isoform is the major form and flow cytometry confirmed good levels on the surface of the SCF ER-Hoxb8 cells. Thus there appears to be a mechanism in SCF ER-Hoxb8 progenitors that suppresses IL-3 signaling via IL-3Rα SP1. It seems likely that this mechanism promotes signaling via the SP2 isoform which may be optimal for IL-3-induced differentiation of GM progenitors. Recent experiments in our group with normal mouse bone marrow cells have confirmed that the SP2 isoform is utilized for the normal differentiation of GM progenitors (J. Chen and I. Young, unpublished).

Cytokines regulate various hematopoietic cell functions through the activation of signal transduction pathways. IL-3 is known to activate many signaling cascades including the Ras/MAPK/Erk pathway, PI3 kinase/Akt pathway and Jak/STAT pathway. In the present work, the IL-3 signaling pathways in SCF ER-Hoxb8 progenitors were compared with those in the widely studied IL-3-dependent cell line Ba/F3. Jak2, Stat5, Erk1/2 and Akt were activated in SCF ER-Hoxb8 progenitors treated with mIL-3. The signaling was shown to be dependent on the IL-3Rα SP2 isoform by using mIL-3 E23A. After lineage maturation, neutrophils again efficiently utilized the SP1 isoform of IL-3Rα for IL-3 signaling. The IL-3 signaling pathways in differentiating SCF ER-Hoxb8 progenitors were very similar to those in Ba/F3 cells which don't differentiate, except for the mIL-3Rα isoform utilized. Clearly, further work is required to elucidate the

critical links between IL-3R signaling and differentiation which are not reflected in the IL-3 signaling pathways as they are currently known.

Although specific transcription factors are believed to control lineage determination during myeloid differentiation they appear to function in conjunction with miRNAs which provide an additional level of control (Zhu and Emerson, 2002; Fatica *et al.,* 2008). In the present work, the levels of miRNAs and the mRNAs for relevant transcription factors were measured in SCF ER-Hoxb8 progenitors comparing cytokine-driven differentiation and proliferation. Significant induction of miR-223, and up-regulation of transcription factors such as C/EBPε, C/EBPβ, PU.1 and GATA-1 were shown in differentiating cells. miR-223 is up-regulated in myeloid differentiation and is a critical inducer of differentiation in human promyelocytic NB4 cells which have been induced to differentiate by ATRA (Fazi *et al.,* 2005). C/EBPβ and PU.1 have been linked to the up-regulation of miR-223 (Fukao *et al.,* 2007).

The upregulation of C/EBPβ and C/EBPε in differentiating SCF ER-Hoxb8 progenitors is particularly interesting. The C/EBP family of transcription factors plays important roles in myeloid differentiation. C/EBPα is expressed in the earliest myeloid cells and required for production of GM progenitors (Zhang *et al.,* 2002). Subsequently, C/EBPβ and C/EBPε are induced during granulocytic differentiation (Scott *et al.,* 1992; Radomska *et al.,* 1998). In acute promyelocytic leukemia, there is a marked decrease in C/EBPα DNA binding activity and treatment of PML cells with ATRA results in induction of first C/EBPβ and then C/EBPε as granulocytic differentiation is restored (Park *et al.,* 1999; Duprez *et al.,* 2000; Duprez *et al.,* 2001). Interestingly, when C/EBPα $^{-/-}$ cells are stimulated with the IL-3 and GM-CSF, but not with ATRA, granulocytic differentiation is also restored with induction of C/EBPβ and C/EBPε (Zhang *et al.,* 2002). Thus, the pathways of steady state granulocytic differentiation,

restoration of differentiation of PML cells with ATRA and IL-3-driven differentiation have a number of features in common. It seems likely that, while the mechanisms involved in the induction of differentiation via these pathways are independent, they share common mechanisms for promoting differentiation.

The miRNA array data suggests that a broader investigation of the miRNAs whose levels are changed with the onset of differentiation is warranted and that IL-3, GM-CSF and SCF signaling may generate unique miRNA profiles consistent with their different differentiation outcomes. Time did not permit further more detailed studies of the miRNA circuits involved using functional approaches such as ectopic expression and antagomirs (Krutzfeldt *et al.,* 2005; Fatica *et al.,* 2008). More detailed understanding of the mechanisms controlling cytokine-driven differentiation are critical for the development of new treatments for allergic inflammation and to improve our understanding of the pathogenesis of AML.

Chapter 6

Final discussion

Blood cell formation involves proliferation of hematopoietic stem cells coupled with progressive maturation and lineage commitment. There is a critical requirement to maintain stem cell numbers by self-renewal which involves replication without differentiation. Leukemic stem cells with abnormal self-renewal capacity are believed to be the critical tumour initiating cells in acute leukemias and appear to arise from normal stem cells or from later progenitors (Passegue *et al.*, 2003; Hope *et al.*, 2004). Although there has been progress on the action of oncoproteins and the genes involved in self-renewal, the mechanisms which control both differentiation and the balance between self-renewal and differentiation are poorly understood.

The focus of this thesis is on myeloid differentiation. Four myeloid differentiation pathways have been recognized: (1) steady state myeloid differentiation, which maintains the normal levels of mature myeloid cells, (2) cytokine-driven or inducible myelopoiesis which generates additional myeloid effector cells during infections or allergic responses, (3) ATRA-induced differentiation of PML cells and (4) CD44-driven differentiation of AML blasts. The aim of the work described in this thesis was to gain a better understanding of IL-3-driven myeloid differentiation and to establish an *in vitro* model for studying mouse myeloid differentiation so that findings can be verified by *in vivo* studies.

Optimal differentiation models for *in vitro* studies should be conditional or inducible so that the onset of differentiation is controlled and detailed studies of the mechanisms can be carried out. It is difficult to establish steady-state differentiation models *in vitro* since it is not possible to accurately reproduce the normal signals provided by the bone marrow niche for self-renewal versus differentiation and when progenitors are maintained *in vitro* on a cocktail of cytokines they rapidly mature. However, knock-out

mice have been useful in giving information on the role of transcription factors in steady-state myeloid differentiation (Coffer *et al.*, 2000; Rosenbauer and Tenen, 2007; Dey *et al.*, 2010). Zhang *et al.*, (2002) derived cell lines resembling immature myeloid progenitors from C/EBPα $^{-/-}$ mice. Conditional expression of C/EBPα induced the C/EBP family members C/EBPβ and C/EBPε and subsequent granulocytic differentiation. Interestingly, similar results were obtained when C/EBPα $^{-/-}$ cells were stimulated with IL-3 and GM-CSF, but not with all-*trans* retinoic acid, supporting the concept of an independent cytokine-induced myeloid differentiation pathway involving C/EBPβ and C/EBPε but not C/EBPα. These findings are in good agreement with the studies of IL-3-induced differentiation of SCF ER-Hoxb8 progenitors discussed below.

Two main differentiation models were used in the work described in this thesis: (1) SCF ER-Hoxb8, a conditionally immortalized granulocyte-macrophage progenitor used for studying mIL-3-driven differentiation and (2) NB4 PML cells, a model for ATRA-induced differentiation. SiRNA knockdown experiments were carried out to explore possible overlap between the steady-state, cytokine-induced, ATRA-induced or CD44-driven differentiation pathways.

The dysfunctionality of IL-3-driven differentiation in AML is supported by the observation that over 80% of AML blasts have high affinity IL-3 receptors but IL-3 does not induce their differentiation (Hsu *et al.*, 1996; Alexandera *et al.*, 2001). Thus, both the cytokine-driven and steady-state differentiation pathways may commonly be blocked in AML. In the work described in Chapter 3, the IL-3R system was investigated in NB4 cells. NB4 is a human promyelocytic leukemia cell line which is not induced to differentiate by hIL-3 or hGM-CSF like a normal promyelocyte (Hsu *et al.*, 1996). However, NB4 differentiates to neutrophils in the presence of ATRA (Lee *et al.*, 2002).

The NB4 cell line carries the t (15; 17) translocation resulting in the joining of the PML oncogene to the gene encoding the RARα (Lanotte *et al.,* 1991).

The data showed that the IL-3R subunits were expressed at a low level in NB4 cells but were otherwise normal suggesting that the block in IL-3-induced differentiation was likely to be downstream of the IL-3R. When the levels of the hIL-3R (hβc with either hIL-3Rα SP1 or SP2) were elevated by ectopic expression, hIL-3-driven differentiation was restored, without ATRA, as shown by morphological analysis and surface marker expression. However, the differentiation achieved was only partial, presumably due to the inhibitory effect of the PML-RARα protein. Although ATRA treatment elevated miR-223 about 5-fold, only a small increase was achieved with IL-3 alone. MiR-223 is a key member of a regulatory circuit involving C/EBPα and NFI-A which is believed to control granulocytic differentiation in ATRA-treated APL cell lines (Fazi *et al.,* 2005).

It has been shown that neutralizing antibodies to hIL-3, hGM-CSF and hG-CSF partially block ATRA-induced differentiation of NB4 suggesting a possible involvement of βc receptor signaling in the process (Matsui *et al.,* 2005). However no synergistic effects between hIL-3 or hGM-CSF and ATRA were detected. Similarly, siRNA knockdown of hβc had no significant effect on NB4 differentiation induced by ATRA. Collectively these results indicate that the mechanisms involved in the induction of differentiation via the IL-3-driven and ATRA-induced differentiation pathways are independent in NB4 cells.

A CD44-induced myeloid differentiation pathway has been shown in AML blasts and NB4 promyelocytic leukemia cells (Charrad et al., 1999; Charrad *et al.,* 2002; Song *et al.,* 2004; Jin et al., 2006) although little is known of the mechanisms involved. The potential involvement of CD44 and its ligand OPN in myeloid differentiation was

explored in the work described in Chapter 4. The CD44-induced differentiation pathway has not been defined in mouse cells. The expression of CD44 and OPN mRNAs was shown in each of four mouse myeloid cell lines undergoing IL-3-induced differentiation. The isoforms expressed were the standard isoforms in each case with no changes detectable between growth and differentiation. In conditionally-immortalized mouse SCF ER-Hoxb8 progenitors no expression of the CD44 ligand OPN was detected in growing cells but the expression of OPN mRNA was detected during differentiation. Further experiments are required to demonstrate the functional significance of the OPN induction demonstrated in the present work.

The expression of CD44 and OPN in NB4 cells undergoing ATRA-induced differentiation was also measured. The expression of CD44 and OPN mRNAs were readily detectable and the levels of expression did not change significantly before and after induction of differentiation by ATRA. Human OPN has three distinct isoforms. OPN-A constitutes the full-length transcript, whereas OPN-B and OPN-C result from alternative splicing (Young *et al.,* 1990; Saitoh *et al.,* 1995; He *et al.,* 2006). DNA sequencing of OPN cDNA clones from NB4 cells showed a change in OPN isoform expression after treatment with ATRA. While parental NB4 cells solely expressed the full length OPN-A isoform, differentiating NB4 cells additionally expressed the OPN-C and OPN-B isoforms. The significance of this finding is not clear, and requires further investigation.

CD44-induced differentiation is as effective as ATRA for the differentiation of promyelocytic leukemia blasts (Charrad *et al.,* 1999). The possible involvement of CD44 in the ATRA-induced and IL-3-induced differentiation pathways was tested by siRNA knockdown of CD44. Neither ATRA-driven nor IL-3-driven differentiation was affected by siRNA knockdown of CD44 in NB4 cells suggesting that CD44 is not a

critical component of these differentiation pathways. Collectively, the results presented in the thesis work suggest that the mechanisms involved in the induction of differentiation via the ATRA-driven, IL-3-driven and CD44-induced differentiation pathways are independent.

The results presented in Chapter 5 show that SCF ER-Hoxb8 progenitors are an excellent model for studying the mechanisms controlling mIL-3-driven differentiation. G-CSF and M-CSF are critical for steady state production of neutrophils and macrophages respectively (Lieschke *et al.,* 1994; Liu *et al.,* 1996; Dai *et al.,* 2002). The work in Chapter 5 showed that the G-CSFR is induced during differentiation in IL-3 and SCF and the M-CSFR is induced during differentiation with GM-CSF. This is an interesting similarity between the cytokine-induced and steady state differentiation pathways to granulocytes and macrophages.

Our group has recently shown the existence of a second isoform of IL-3Rα (designated SP2) which has distinctive binding and signaling properties (Chen *et al.,* 2009; Mirza *et al.,* 2010a) relative to the originally described isoform (designated SP1). An important issue is to define the biological roles of the two isoforms. The studies reported in Chapter 5 revealed a specific role for the mIL-3Rα SP2 isoform in the differentiation of the SCF ER-Hoxb8 progenitors. Recent structure-function studies in our group have shown there are distinct epitopes on IL-3 and the β receptors which are utilized by mIL-3Rα SP1 and SP2 isoforms (Mirza *et al.,* 2010a). It seems likely that that the respective high affinity complexes have different relative orientations of the receptor subunits resulting in different signaling properties. An IL-3 mutant, mIL-3 E23A, has been characterized which is only active with the IL-3Rα SP1 isoform and can be used to demonstrate dependence on IL-3 signaling via the SP2 isoform. In the work described in Chapter 5 it was shown that SCF ER-Hoxb8 progenitors did not grow with mIL-3

E23A. This indicates that these cells predominantly use the IL-3Rα SP2 isoform for mIL-3 signaling. Interestingly, however, sequencing analysis and Western blotting showed expression of normal IL-3Rα SP1 in SCF ER-Hoxb8. In fact the SP1 isoform is the major form and flow cytometry confirmed good levels on the surface of the SCF ER-Hoxb8 cells. Thus there appears to be a novel mechanism in SCF ER-Hoxb8 progenitors that suppresses IL-3 signaling via IL-3Rα SP1. This suggests that IL-3 signaling via IL-3Rα SP2 is required for optimal differentiation of GM progenitors. Recent experiments with normal mouse bone marrow cells have confirmed that the SP2 isoform is also utilized for the optimal differentiation of GM progenitors from bone marrow (J. Chen and I. Young, unpublished).

Interestingly, autocrine IL-3 production occurs reasonably commonly in mouse leukemias arising from the expression of oncoproteins. For example, Hoxb8 expression in bone marrow cells allows the ready isolation of IL-3-dependent cell lines which are blocked in differentiation (Perkins and Cory, 1993). Some of the spontaneous leukemias arising from Hoxb8 expression are autocrine for IL-3 and simultaneous over-expression of both Hoxb8 and IL-3 rapidly yields a transplantable myeloid leukemia (Perkins *et al.*, 1990).

In human AML, IL-3Rα is overexpressed on leukemic stem cells and blasts and plays a potential role in the pathogenesis of this disease. It is currently being developed as a target for anti-leukemia therapy (Testa *et al.*, 2004). It was recently reported that an anti-IL-3Rα chain (CD123)-neutralizing antibody (7G3) targeted AML-LSCs, impairing homing to bone marrow (BM) and activating innate immunity of nonobese diabetic/ severe-combined immunodeficient (NOD/SCID) mice. 7G3 treatment profoundly reduced AML-LSC engraftment and improved mouse survival (Jin *et al.*, 2009). In view of the specific role revealed for IL-3Rα SP2 in mouse myeloid differentiation it will be

of interest to see if this also is true of human GM progenitors and whether there are any derangements in AML.

IL-3 is known to activate the Ras/MAPK/Erk, PI3 kinase/Akt and Jak/Stat signaling pathways. Definition of the IL-3 signaling pathways has resulted from work with IL-3-dependent lines like Ba/F3 which grow continuously on IL-3 but do not differentiate. In the present work, the IL-3 signaling pathways in SCF ER-Hoxb8 progenitors undergoing IL-3-driven self-renewal or differentiation were compared with those in Ba/F3. It was shown that Jak2, Stat5, Erk1/2 and Akt were activated in SCF ER-Hoxb8 progenitors treated with mIL-3. The signaling was shown to be dependent on the IL-3Rα SP2 isoform using mIL-3 E23A. The IL-3 signaling pathways in differentiating SCF ER-Hoxb8 progenitors were very similar to those in Ba/F3 cells, except that these cells specifically signaled via the mIL-3Rα SP2 isoform whereas the Ba/F3 cells clearly utilized mIL-3Rα SP1. Further work is required to elucidate the critical links between IL-3R signaling and differentiation which are not reflected in the IL-3 signaling pathways as they are currently known.

Recently, Doulatov *et al.*, (2009) reported evidence that the transcription factor PLZF is a critical regulator of myeloid differentiation restricting proliferation and differentiation of myeloid progenitors. PLZF represses transcription factors involved in myeloid differentiation including GFI-1, C/EBPα and LEF1. Induction of MEK1/2 by IL-3 leads to nuclear export and inactivation of PLZF which augments mature cell production. Future investigation of the role of PLZF and of the mIL-3Rα isoforms in regulating IL-3-driven myeloid differentiation would be of great interest.

Transcription factors are believed to control lineage determination during myeloid differentiation and appear to function in conjunction with miRNAs (Zhu and Emerson,

2002; Fatica *et al.,* 2008). A common myeloid precursor gives rise to mature granulocytes and monocytes and their production depends on the activity of several transcription factors. A crucial role in the initial steps of myelopoiesis is played by C/EBPα and PU.1. These two factors are responsible for the transcriptional activation of miR-223 and miR-424 respectively that down-regulate the common target NFI-A. NFI-A counteracts the differentiation of both granulocytes and monocytes, thus its down-regulation is important for myelopoiesis to proceed. An interesting autoregulatory loop appears to control granulocytic differentiation of APL cells: C/EBPα activation produces the displacement of NFI-A from the miR-223 promoter and its up-regulation. In cascade, miR-223 acts by repressing NFI-A thus removing it from the competition with C/EBPα and maintaining sustained levels of miR-223 expression (Fazi *et al.,* 2005). In the present work, the levels of miRNAs and the mRNAs for relevant transcription factors were measured in SCF ER-Hoxb8 progenitors comparing self-renewal with cytokine-driven differentiation. With the onset of differentiation, significant induction of miR-223, and up-regulation of transcription factors such as C/EBPε, C/EBPβ, PU.1 and GATA-1 was observed.

The data in Chapter 5 showed upregulation of C/EBPβ and C/EBPε in differentiating SCF ER-Hoxb8 progenitors. In steady state myeloid differentiation, C/EBPα is expressed in the earliest myeloid cells and required for production of GM progenitors (Zhang *et al.,* 2002). Subsequently, C/EBPβ and C/EBPε are induced during granulocytic differentiation (Scott *et al.,* 1992; Radomska *et al.,* 1998). Similarly, treatment of promyelocytic leukemia cells with ATRA gives induction of first C/EBPβ and then C/EBPε as granulocytic differentiation is restored (Duprez *et al.,* 2000; Duprez *et al.,* 2001; Park *et al.,* 1999). Also, when C/EBPα $^{-/-}$ myeloid progenitors are stimulated with IL-3 and GM-CSF, granulocytic differentiation is restored with

induction of C/EBPβ and C/EBPε (Zhang *et al.,* 2002). Thus, although the mechanisms leading to induction of differentiation via the pathways of steady state granulocytic differentiation, ATRA-induced differentiation of PML cells and IL-3-driven differentiation are independent they have in common the induction of C/EBPβ, C/EBPε and miR-223 suggesting common mechanisms for the later steps.

Previous studies have shown different roles for C/EBPβ and C/EBPε in myeloid differentiation. Yamanaka *et al.,* (1997) reported C/EBPε is required for the terminal differentiation and maturation of granulocytes from studies of C/EBPε-deficient (C/EBPε $^{-/-}$) mice. Recently, it has been shown that, in the absence of C/EBPε, there is not only incomplete differentiation of granulocytes, but myelopoiesis is disrupted with the appearance of an intermediate cell type with monocyte and granulocyte features, and the neutrophils have abnormal chemotaxis (Halene *et al.,* 2010). C/EBPε $^{-/-}$ mice also has defective eosinophil differentiation (Bedi *et al.,* 2009). C/EBPβ is upregulated during differentiation of myeloid cells (Natsuka *et al.,* 1992; Scott *et al.,* 1992), but no defects in granulopoiesis are apparent in C/EBPβ $^{-/-}$ mice (Screpanti *et al.,* 1995). However, C/EBPβ is essential in granulopoiesis specifically in conditions of stress, in which various factors, including cytokines and pathogens, stimulate the mobilization of granulocyte progenitors (Hirai *et al.,* 2006).

The miRNA array data in Chapter 5 suggests that a broader investigation of the miRNAs whose levels are changed with the onset of differentiation is warranted. It appears that IL-3-, GM-CSF- and SCF-driven differentiation may generate unique miRNA profiles consistent with the different composition of mature cell types that are produced by the three pathways. More detailed studies of the miRNA circuits involved in IL-3-driven differentiation using functional approaches such as ectopic expression

and antagomirs (Krutzfeldt *et al.,* 2005; Fatica *et al.,* 2008) is also required. Antagomirs, a novel class of chemically engineered oligonucleotides, are powerful tools to silence specific miRNAs *in vivo* and may represent a therapeutic strategy for silencing miRNAs in disease (Krutzfeldt *et al.,* 2005). Recently, it has been shown that antagonism of miRNA-126 suppresses the effector function of Th2 cells and the development of allergic airways disease in mice (Mattes *et al.,* 2009). The work described in Chapter 5 provides the foundation for future studies which could lead to miRNA directed strategies for controlling allergic inflammation. More detailed understanding of the mechanisms controlling cytokine-driven differentiation may also improve our understanding of AML.

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